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Interaction of 60S core ribosomal proteins from a ciliated protozoan with mature and precursor ribosomal RNAs

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INTERACTION OF 60S CORE RIBOSOMAL PROTEINS FROM A CILIATED PROTOZOAN WITH MATURE AND PRECURSOR RIBOSOMAL RNAs

By

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LIST OF ABBREVIATIONS

lrRNA	large ribosomal RNA
LS	large 60S ribosomal subunit
SS	small 40S ribosomal subunit
YTR	yeast SP6 transcript with entire L25 binding site
pGB	*T.thermophila* T7 transcript [precursor]
TCP	*T.thermophila* core ribosomal proteins
YL25	yeast L25 protein
RNP	ribonucleoprotein particle
β-ME	β-mercaptoethanol

ABSTRACT

Ribosomal proteins participate in the processing and maturation of precursor rRNAs to form functional subunits. The interaction of an early binding 60S ribosomal protein L25 with the 23S/26S/28S rRNAs is well documented. The interaction of these protein homologues from prokaryotes, eubacteria, and archaebacteria with the 23S/26S/28S rRNA is highly conserved. Its binding site has been mapped to Domain III of the large rRNA [lrRNA] in which the "hidden break" occurs in insects [*D.melanogaster*] and ciliated protozoans [*T.thermophila*]. In these species, excision of nucleotides within the hidden break results in the formation of an α and β moiety, that are held together by hydrogen bonds. L25 homologues have been isolated from a series of prokaryotic

organisms [e.g. *E.coli* & *B.stearophilus*], however the only eukaryote from which L25 has been identified is yeast. Yeast is an organism in which the hidden break is not present. Heterologous binding studies have shown that yeast L25 binds preferentially to *T.thermophila* mature 26S rRNA while no binding was observed with the precursor rRNA transcript, in which the gap sequences were present [Ware, submitted]. Hence, it was proposed that perhaps processing at this site [variable V9 region], may be a prerequisite for L25 binding in protozoans. It is also possible that differences between L25 binding interactions exist in organisms with the break [*T.thermophila*] and without the break [yeast]. In order to study these binding differences in these organisms, an attempt was made to isolate the L25 homologue form *T.thermophila*. Using extraction methods successful for yeast, three 15 Kd core ribosomal proteins were isolated

from *T.thermophila*. *T.thermophila* core proteins demonstrated a preferential interaction with mature 26S rRNA. However, no binding was observed with the precursor transcript. The core proteins appear to protect two unique fragments on the 26S rRNA as determined by limited RNase T1. Inetraction of core proteins with yeast transcript containing the entire L25 binding site suggests that an L25-like homologue may be present. This will be confirmed by RNA sequencing of the protected fragments.

CHAPTER I

INTRODUCTION

Ribosomes are sophisticated complex ribonucleoprotein particles that have a primary role in protein synthesis. Elucidating their structure, function, and assembly into mature subunits presents a unique challenge to molecular biologists, since they are composed of nearly sixty different molecules [proteins and rRNAs]. Ribosomes consist of two subunits, the large subunit [LS] and the small subunit [SS] which differ in size and sedimentation coefficients in prokaryotes [50S/30S] and eukaryotes [60S/40S], [see Table 1. for

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Comparison of ribosomal subunits]. This size disparity has been attributed to the increased rRNA size as well as an increase in number of ribosomal proteins in eukaryotes. However, eukaryotic ribosomes do not differ functionally from prokaryotic species in a fundamental manner [refer to Tables 2 & 3]; that is, they both perform the same function by similar biochemical means. Hence the critical question posed is why then are eukaryotic ribosomes larger and have a greater number of proteins than their prokaryotic counterparts ? I will focus this discussion to the structure and biogenesis of eukaryotic ribosomes.

	Prokaryotes	Eukaryotes
Source	E. coli	Rat liver
Ribosomes		
Sedimentation	70S	80S
Mass (KDa)	2520	4420
Large subunit		
a) RNA components	23S = 2904b 5S = 120b	28S = 4700b 5.8S = 160b 5S = 120b
Proportion of Ribosome mass	70%	65%
b) Proteins	31 polypeptides	49 polypeptides
Proportion of Ribosome mass	30%	35%
Small subunit		
a) RNA components	16S = 1541b	18S = 1900b
Proportion of Ribosome mass	60%	50%
b) Proteins	21 polypeptides	33 polypeptides
Proportion of Ribosome mass	40%	50%

Table 1. : COMPARISON OF EUKARYOTIC AND PROKARYOTIC RIBOSOMES. [Abbreviation : b = bases ; adapted from Lewin , 1985]

RIBOSOMAL BIOGENESIS

Ribosomal biogenesis involves the processing of precursor rRNA as well as assembly of ribosomal proteins to form mature functional subunits [see fig 1. for summary of ribosomal biogenesis]. Processing of rRNAs is an elaborate procedure involving initial transcription of the rDNA repeat [see fig.2.] to form a single 45S rRNA transcript which undergoes a cascade of processing steps to form mature functional rRNAs.

Ribosomal RNA genes have been localized to a distinct nuclear organelle called the nucleolus. Eukaryotic rRNA processing is similar from mammals to fungi; a single transcript is produced in the nucleolus by a species-specific polymerase,

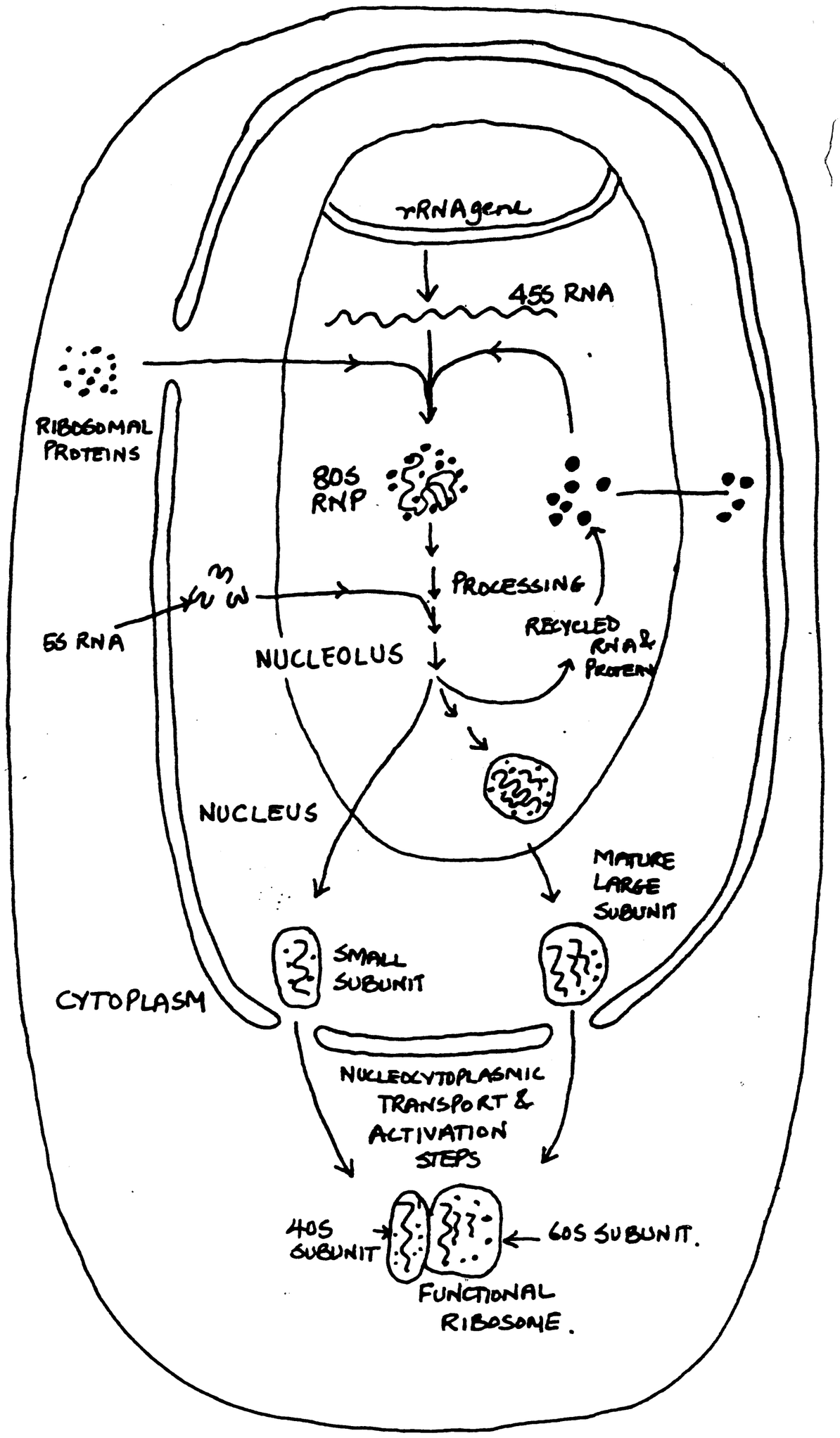

Fig.1. : SCHEMATIC REPRESENTATION OF THE OVERALL BIOGENESIS OF RIBOSOMES. [taken from Molecular Biology of the Cell , Alberts , 1985]

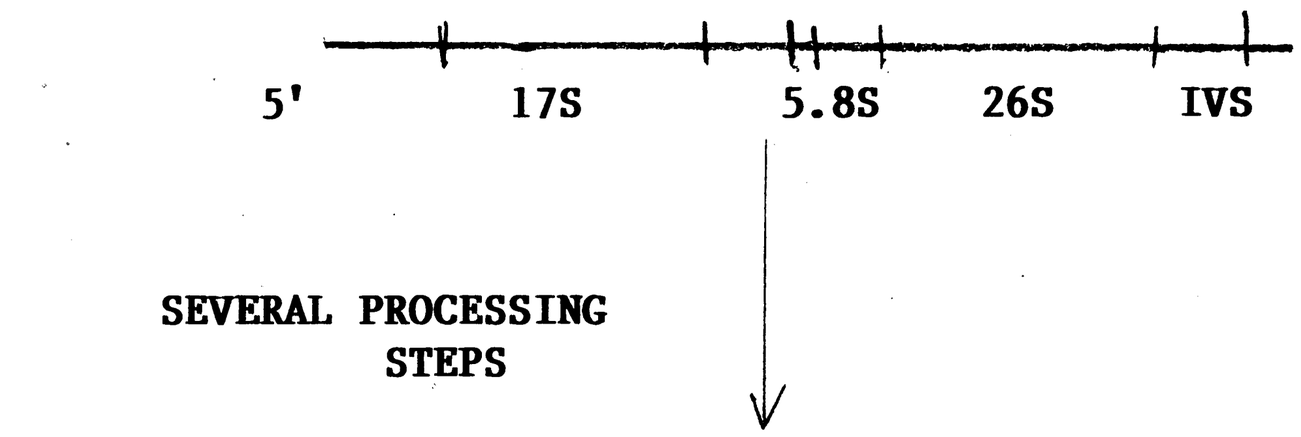

Fig.2. : GENETICS OF TETRAHYMENA RIBOSOMES. [a] Organization of rDNA repeat ; [b] Ribosomal RNA processing. [taken from Molecular Biology of Ciliated Protozoans Ed. Gall ; 1989]

methylation occurs either during or after transcription, followed by sequential cleavage at specific sites to form three stable RNAs; 18S; 28S and 5.8S rRNAs. The first cleavage is endonucleolytic resulting in the formation of a 3' end that then excises 7 nucleotides exonucleolytically [see fig.3.]. Pulse chase experiments have shown that a unique set of proteins larger than ribosomal proteins, are associated with the RNP but do not pass out into the cytoplasm [see fig.4. ; Warner and Kumar, 1972]. It has been hypothesised that since these proteins are re-utilised during ribosome formation, they may be involved in the processing of the 45S precursor transcript.

There is evidence that small nuclear RNPs [snRNPs] in vertebrates [for example U3 snRNP] and in lower eukaryotes [e.g.,yeast snRNA 3,4,5,8,9,10,17,128 & 190] bind to precursor rRNAs in the nucleolus [Tollervey & Fournier 1989]. It has been suggested that these snRNAs might be components of early processing events of the pre-rRNAs in the nucleolus, in a manner similar to spliceosomes for mRNAs. Alternatively, they could also play a role in the correct folding of pre-rRNAs, ribosomal assembly, or even nucleocytoplasmic transport. Of the nine snRNAs identified in yeast, only snRNA 17 appears to be essential for yeast viability, implying that snRNA 17 may be playing a vital role in some ribosomal assembly or processing step.

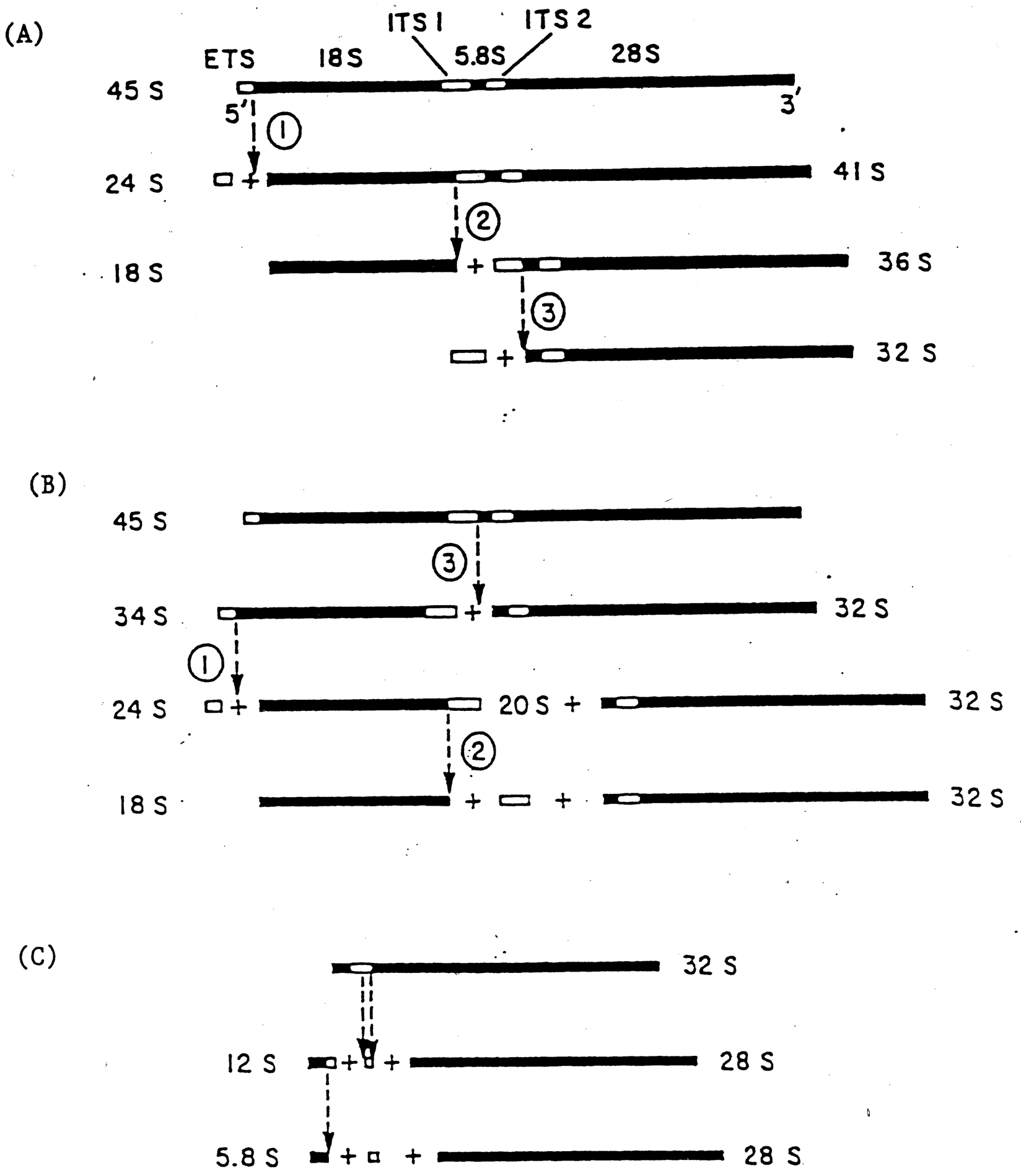

Fig.3. : EUKARYOTIC RIBOSOMAL RNA PROCESSING . Processing scheme for the maturation of eukaryotic rRNAs . White boxes are transcribed spacers ; black boxes are mature RNA regions. [a] Separation of 18 S rRNA from 32 S rRNA ; in HeLa cell at 38 ° C [1] and 33 ° C [2] ; [b] Processing to 28 S rRNA and 5.8 S rRNA. [taken from de Jansverin and Jacq, 1989]

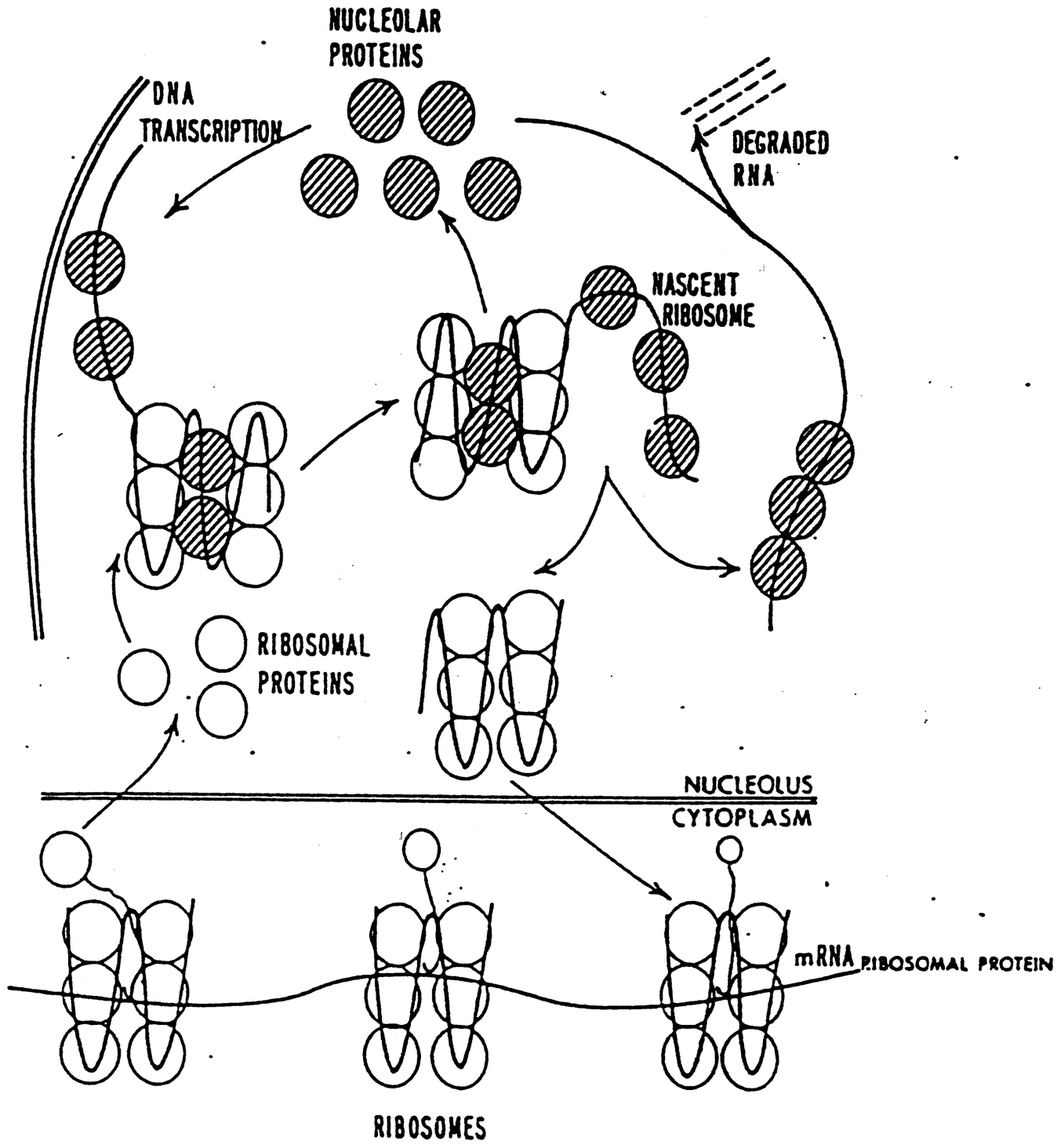

Fig.4. : ROLE OF NUCLEOLAR PROTEINS IN RIBOSOME FORMATION. Topological orientation of proteins is arbitary. [taken from J.R. Warner; Ribosomes Cold Spring Harbor Laboratory,1974].

The most complex rRNA processing has been observed in certain insects [*Sciara coprophila* ; *Bombyx mori* ; *Drosophila melanogaster*] and protozoans [*Tetrahymena thermophila* (*T.thermophila*)], in which additional processing events occur in the 26S rRNA. The occurrence of a hidden break in the center of the 26S / 28S lr RNA has been demonstrated in protozoans [*T.thermophila*, V.C. Ware] and insects [see Table 4; *Sciara coprophila*, V.C. Ware 1985 ; *Drosophila melanogaster* (*D.melanogaster*), de Lansverin and Jacq 1984; *Bombyx mori*, Fujiwara and Ishiwara 1986]. Denaturation and pulse-chase experiments have shown that the occurrence of the hidden break is indeed a true maturation step and not an artifact of RNA isolation. This processing event [also called "gap processing"] occurs in insects and protozoans while in lower eukaryotes [yeast] and vertebrates

THE DISTRIBUTION OF THE HIDDEN BREAK IN THE 26S RNA OF ANIMALS

SPECIES	COMMON NAME	HIDDEN BREAK
Euglena gracilis		+
Tetrahymena thermophila		+
Rana nigrimaculata	Frog	-
Mus molossinus	Mouse	-
Homo sapiens	Man	-
Fasciola hepatica	Liver fluke	+
Ascaris lambricoides	Round worm	-
Bombyx mori	Silk moth	+
Drosophila melanogaster	Vinegar fly	+
Sciara coprophila	Fungus fruitfly	+

TABLE 4. : LIST OF ORGANISMS IN WHICH THE HIDDEN BREAK OCCURS.

(adpated from H. Ishikawa.; 1977)

[*X. laevis*] this phenomenon does not occur [see fig.6]. Fragmentation has been documented in the 26S-28S as well as the 17S rRNAs in other organisms [dinoflagellates have hidden breaks in V3] and even some prokaryotic organisms [e.g. *Salmonella*]. Some protozoans also exhibit a break in the 5.8S rRNA [e.g. *T. thermophila*, see fig.5B].

Gap processing results in the cleavage of the 26S-28S rRNA into 2 moieties, the 26S α and 26S β that are held together by hydrogen bonds [see fig.5A]. The site of cleavage has been mapped to a eukaryotic specific variable region [V9] in the central domain [DIII] of the 26S-28S rRNA. It is believed that these processing events occur late in the maturation of ribosomal subunits. The

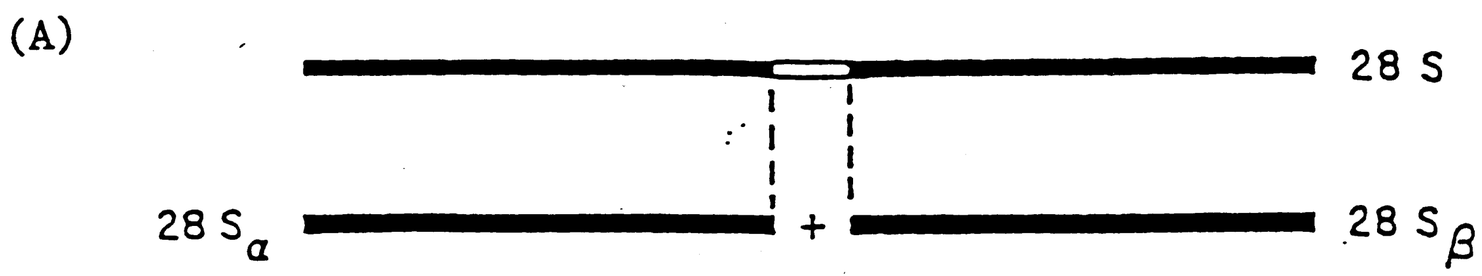

Fig.5 : ADDITONAL PROCESSING STEPS IN INSECTS AND CILIATED PROTOZOANS. [a] Processing of the hidden break in 26 S / 28 S rRNAs. [b] Fragmentation in 5.8 S rRNA . [taken from de Lansverin and Jacq, 1989]

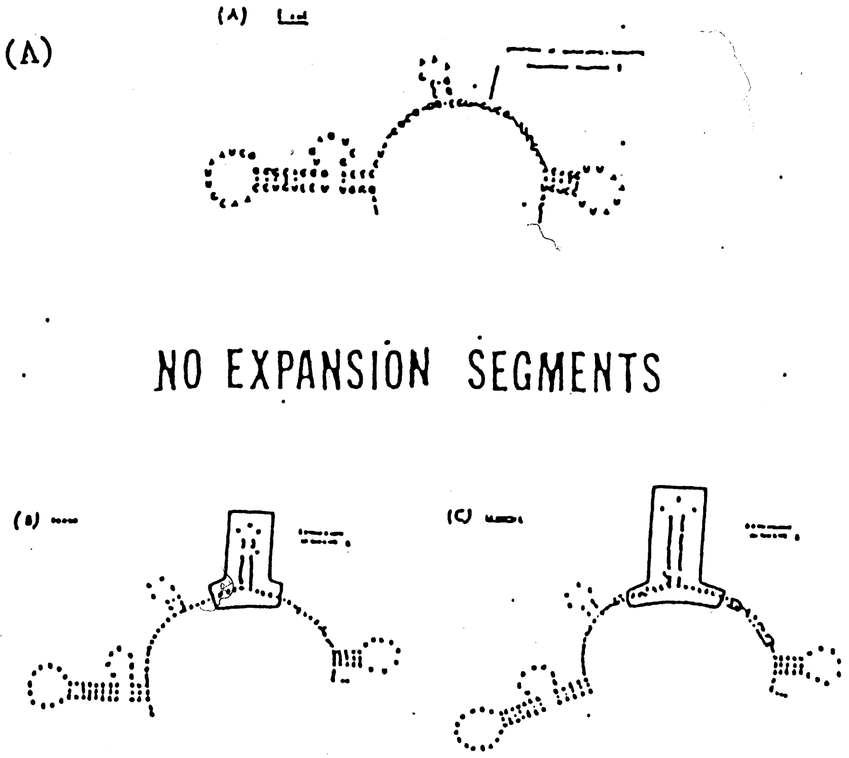

Fig.6 : COMPARISON OF THE CENTRAL DOMAIN FROM PROKARYOTES AND EUKARYOTES. [a] *E.coli* ; [b] eukaryotes with no gap processing and [c] eukaryotes with gap processing [V.C.Ware, personal communication].

significance or the mechanism of gap processing is still unclear.

Ribosomal RNAs are divided into secondary structure domains that consist of conserved domains and eukaryotic specific variable/divergent domains. The secondary structure of Divergent / Variable [V9] region consists of two important features: namely they can be folded into an independent structure that does not affect folding of the conserved common core and secondly, they are always inserted in the same three specific sites of the common core* [D7a / D7b / D7c]. D7a has been localized to helices J & I and is folded into two consecutive helices [Helix D7a 1 and Helix D7a 2]. Helix D7a 1 has a smaller stem [2 - 4 bp] enclosed by a terminal loop of 3 - 6 nucleotides. Helix D7a 2 is variable in size and never forms a branched structure. The target for

unique processing events in insects and protozoans has been localized to the D7a domain while the D7a 2 helix which is A + U rich is always excised during processing [see fig.7, de Lansevrin and Jacq,1989].

D.melanogaster 26S rRNA contains a 890 base pair sequence in its Central Domain [DIII] in which a break is created by nuclear processing events. The sequences which are processed out have been shown to be very A + U rich as compared to the remaining sequences. The G + C content for the D7a / D7b regions appear to be related in organisms that do not gap process. However there is considerable difference in G + C content in organisms that process out the gap sequences. It appears that there exists a common [ATAATT] hexamer between *Sciara coprophila* & *D.melanogaster*, which is absent in *X. laevis* and *yeast.* Therefore conservation of sequence at the

begining of the 26S - 28S β might imply specificity for cleavage.

In the ciliated protozoan *T.thermophila*, the hidden break has been localised to the variable V9 region, in which insects [Drosophila and Sciara] exhibit gap processing. However, *T.thermophila* only excises 3 nucleotides from the V9 region, Ware and Sherman, submitted [see fig.5A]. In addition to gap processing, *T.thermophila* also exhibits processing of its rRNA intron, at an earlier time in the processing pathway [see fig.8]. Excision of the 26S rRNA intron has been shown to be a self-splicing event and the exact mechanism has been well characterized [Cech et al 1989]. The significance or the mechanism of the gap region in *T.thermophila* still remains a puzzle. It is unknown if processing of the gap region is a self excision event or not.

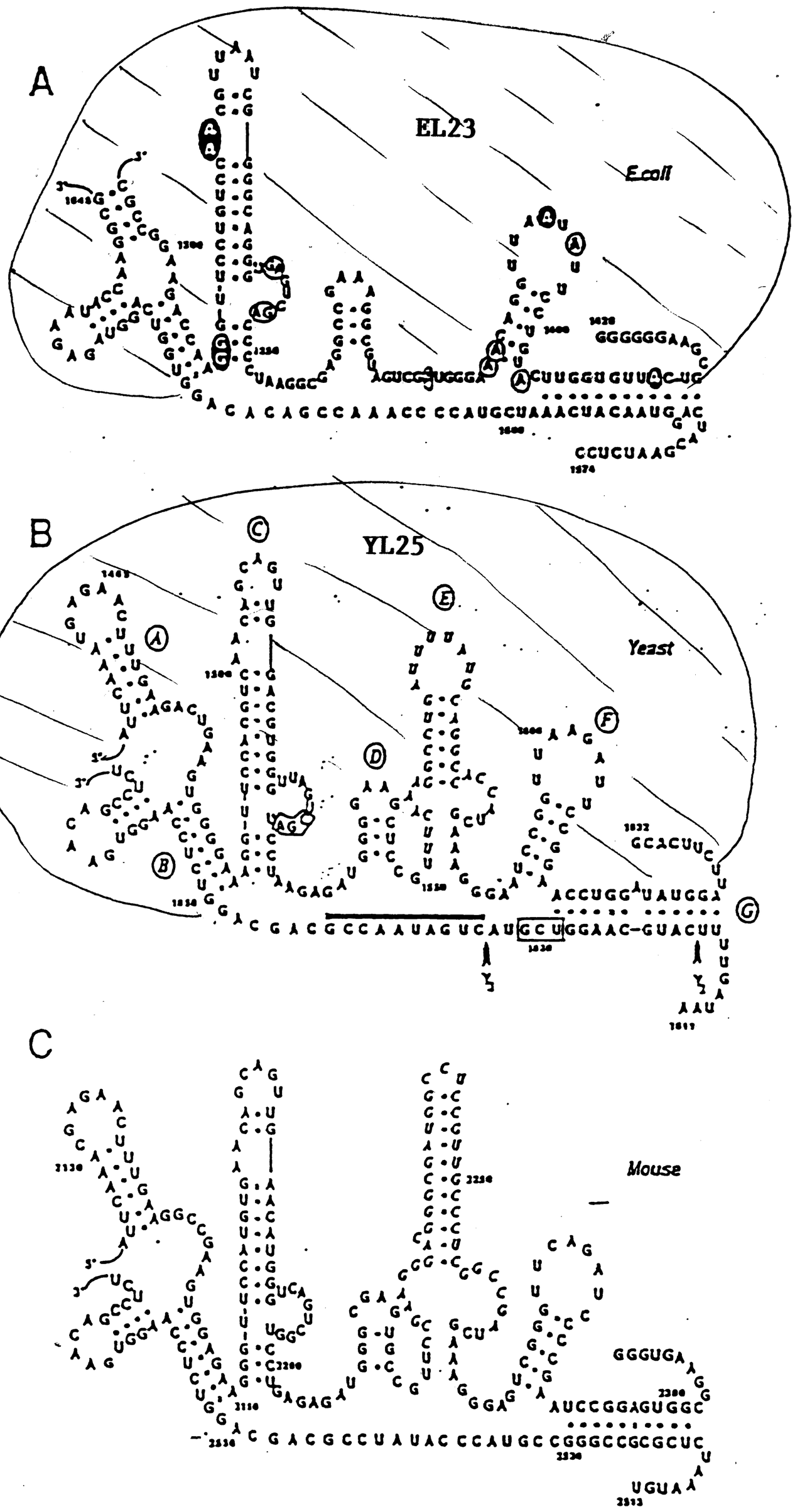

Fig.7 : COMPARISON OF L25 BINDING SITE IN *E.coli* [a] ; yeast [b] and mouse [c]. [taken from El-Baradi et al. 1985]

In summary gap processing events have resulted in the cleavage of phosphodiester bonds as well as excision of nucleotides from the precursor molecules. Cleavage results in the formation of two moieties, 26S α and 26S β , which are held together by hydrogen bonds. It is possible that in evolutionarily similar species, a size limitation could induce processing in the Variable V9 region. It is possible that cleavage may induce a conformational / structural change in the central domain, which may allow for assembly of early binding ribosomal proteins and their subsequent maturation.

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IRREGULAR

PAGINATION

RNA - PROTEIN INTERACTION

Ribosomal assembly involves the simultaneous interaction of proteins with rRNA species to form mature subunits [see fig.9]. Each subunit is assembled with ribosomal proteins and rRNAs into a complex three-dimensional structure that is held together by non-covalent interactions. Ribosomal proteins have an auxillary role in facilitating the proper functioning of rRNA by mediating a shift in conformation of functional sites and inducing or stabilizing the structure of the rRNAs [J.H. Warner ,1975].

A model for ribosomal assembly was proposed by Warner et al [1984], [see fig.9]. Transcription of rRNA occurs freely in the nucleolus, followed by

simultaneous entry of new ribosomal proteins into the nucleolus. These ribosomal proteins can bind to sites on the pre-rRNA. However, binding is not permanent; that is, one protein may move from one RNA to another [Warner at al,1984]. Therefore precursor RNA molecules compete for the limiting ribosomal protein. Ultimately the pre-rRNA with a complete set of ribosomal proteins can be cleaved by a nuclease and exported out into the cytoplasm , as an intact mature subunit.

Ribosomal assembly however is not accomplished in an entirely universal manner. There are subtle differences between prokaryotic, eukaryotic, and organellar ribosomal assemblies. Eukaryotic ribosomes are not assembled on mature 18S & 28S rRNAs but instead initiation of assembly occurs with

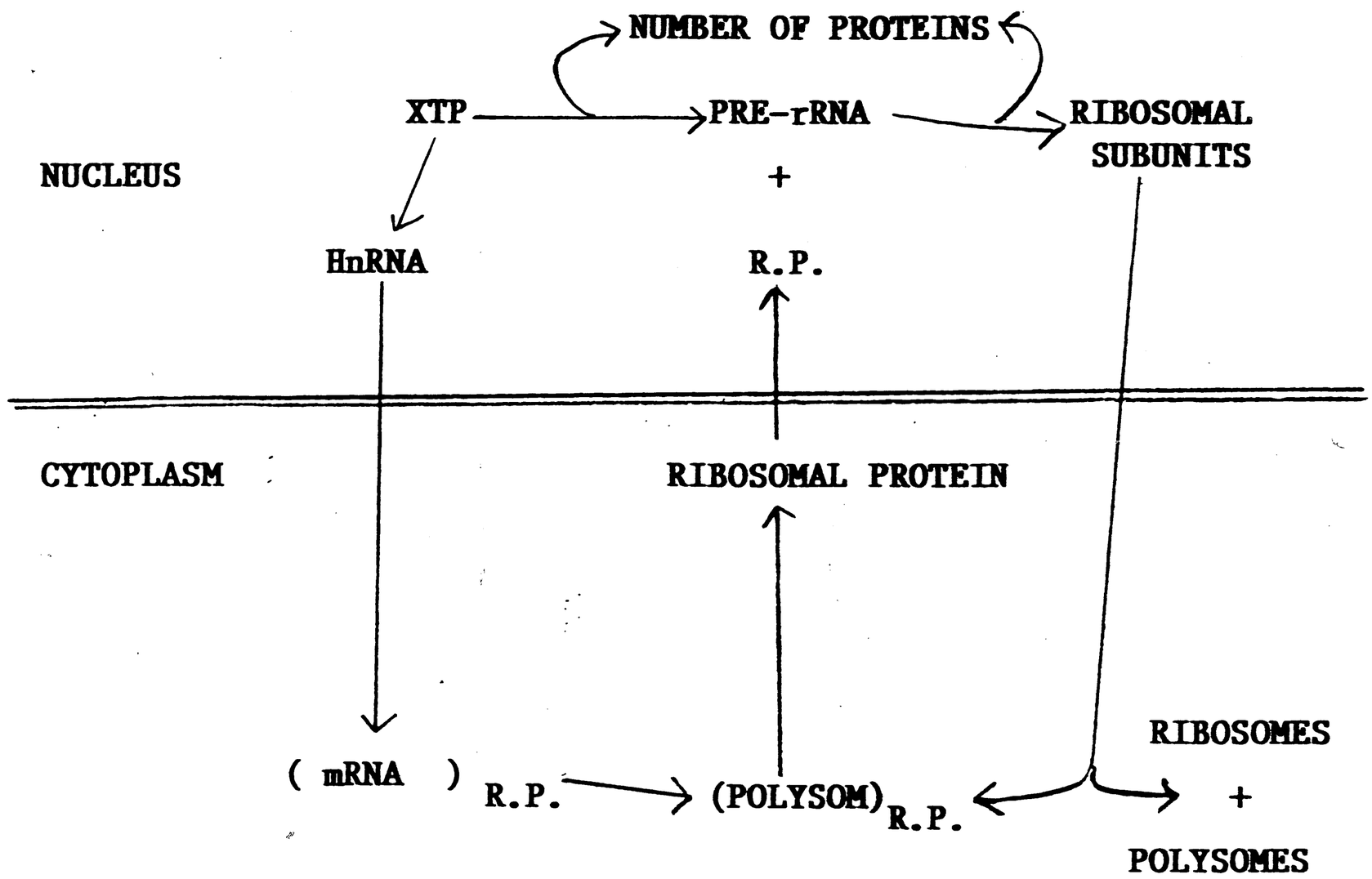

Fig.9 : SCHEMATIC REPRESENTATION OF RIBOSOME SYNTHESIS AND ASSEMBLY IN EUKARYOTES. [taken from J.R. Warner, Ribosomes Cold Spring Harbor Laboratory, 1975]

45S pre-rRNA. The 45S RNA may possess information required for proper assembly. Mitochondrial and chloroplastic ribosomes are distinctly different from their counterparts in the cell sap. There are a few similar or structurally related proteins between organellar and cellular ribosomes, suggesting that some proteins may be synthesized in the cytoplasm and upon entry into the organelle undergo further maturation steps [Stern; Noller and Powers,1989].

Kumar & Warner [1971] have demonstrated in yeast temperature sensitive mutants that unless ribosomal proteins are "fixed" in the nucleolus by attachment to precursor rRNA, they are forced to exit the nucleus and are eventually destroyed. This suggests that there must exist some mechanism by which only the entry of newly synthesized ribosomal proteins are permitted to assemble into mature

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subunits. Clearly there exists some mechanism by which a balance is maintained between the transcription and the rate of processing of ribosomal proteins. For every new ribosome assembled, an old ribosome is degraded [Weber 1972] suggesting that the fate of ribosomal degradation is well regulated.

The spatial orientation of 60S proteins with the 26S rRNA has been determined using protein-protein cross linking experiments [Xiang and Lee, 1990]. Cross-links were generated by treatment of intact 60S subunits with 3,3'-dithiobisproprionimidate and the resulting cross-links were analysed by 2D-diagonal SDS-polyacrylamide gels. Seventeen cross-linked pairs were obtained out of which several ribosomal proteins were involved in multiple cross-linking. It

was assumed that these proteins might serve as foci upon which subsequent assembly occurs. These results indicate that L25 and L29 form associations with 6 and 5 proteins respectively, suggesting that these proteins may be centrally located within the 60S subunit. In yeast, early assembly proteins have been discerned based on their association time with the mature rRNA [Planta,1978]. Cross-linking analysis has shown that 7 out of the 17 cross-linked early assembly protein pairs are clustered [L3-L4 ; L17-L34 ; L26-L29 ; L26-L34 ; L29-L33 ; L29-L38 ; L33-L34]. These clustered proteins form foci on which assembly occurs. L25 and L29 form associations with these cross-linked pairs [Xiang and Lee, 1990].

Ribosomal proteins of the small and large subunit have been isolated in a variety of eukaryotic organisms [refer to Table 5. for summary

of eukryotic ribosomal proteins]. The two most extensively studied eukaryotic ribosomal proteins systems have been for mouse and yeast. Table 6. lists the molecular weights of isolated ribosomal proteins of the 40S and 60S subunits from rat liver.

Ribosomal proteins have been implicated in mediation of subunit assembly and maturation. They are synthesized in the cytoplasm and are required to be imported back into the nucleolus where their assembly occurs [see fig.9].

The most tightly bound 60S ribosomal proteins are L25 > L4 > L8 > L10 > L12 > L16 . These proteins appear to be the first proteins that are assembled onto the 26S rRNA [El-Baradi et al, 1984]. By far the clearest and most extensively studied ribosomal protein / RNA interaction has been the association

SOURCE OF RIBOSOMES	NUMBER OF PROTEINS 40S	60S	80S
RAT LIVER	30	40	70
RAT MUSCLE	31	38	69
RABBIT LIVER	30	38	68
YEAST	30	50	80
FROG	29	–	–

Table.5 : RIBOSOMAL PROTEIN DISTRIBUTION IN EUKARYOTIC RIBOSOMAL SUBUNITS. [taken from Wool, I.G. and Stoffler, G. Ribosomes Cold Spring Harbor Laboratory, 1975]

MOLECULAR WEIGHTS OF 60S RIBOSOMAL PROTEINS OF T.THERMOPHILA

60S

PROTEIN NUMBER	MOLECULAR WEIGHT
L2	56
L4	56
L1	45
L5	45
L3	40
L6	41
L7	39
L8	25
L13	24
L10	22
L12	22.5
L15	23.5
L24	22.5
L18	21.5
L25	21.5
L19	20
L28	21
L16	19.5
L21	17.5
L26	17.5
L30	16
L26	17.5

Table.6 : MOLECULAR WEIGHTS OF RAT LIVER RIBOSOMAL PROTEINS OF THE SMALL AND LARGE SUBUNITS. [taken from Wool, I.G. and Stoffler, G. Ribosomes Cold Spring Harbor Laboratory, 1975]

of L25 with the 26S rRNA in yeast. L25 homologues have been isolated from a variety of organisms including *E.coli* , *B. stearophilus* [Raue,1989]; however the only eukaryote from which L25 has been isolated has been yeast.

The L25 binding site on the 23S / 26S rRNAs is highly conserved among prokaryotes, eubacteria, archaebacteria and eukaryotes. This conserved ribosomal protein binding site is interrupted by the site in which the hidden break occurs in some organisms e.g. *T.thermophla*. The significance of this observation is unknown.

The binding site for yeast [YL25] exhibits a very strong structural homology to its counterpart in *E.coli* EL23 [see fig.7]. RNase T1 protection experiments have localized the binding site for L25

to be within Domain III [1465 - 1632 & 1811 - 1861 bases] of the lr RNA. The structure of the binding site is composed of 2 main fragments, the 25 nucleotides [nts] and 105 nts which are separated by 172 nts. Zimmermann et al [1980] showed that EL23 doesnot bind to yeast 26SrRNA, which poses a question whether the structural differences between YL25 and EL23 are detrimental to prokaryotic binding but tolerable in a eukaryotic situation. Heterologous filter binding assays have shown that E.coli and yeast L25 homolgues are capable of binding to 23S-26S rRNAs, while YL25 was incapable of binding to mouse 28S rRNA [see fig.7; El-Baradi et al, 1987]. EL23 can specifically recognize and interact with the yeast L25 binding site on the 26S rRNA. The use of in vitro rRNA transcripts from cloned rDNA regions was exploited in yeast to

determine the exact structure and sequence of the L25 binding site. An in vitro SP6 transcript pSY26XH from yeast was used in a nitrocellulose filter binding assay. It was shown that YL25 / EL23 bind equally well to the in vitro yeast transcript [El-Baradi et al, 1987]. Re-binding experiments have shown that the binding site is composed of two regions separated by 150 nts [El-Baradi et al 1987]. The putative contact points of the protein for the rRNA have been localized to the 5' proximal protected area. Using deletion mutants the sequence between nucleotides 1819 -1833 was determined to be essential for YL25 / EL23 binding, while sequences immediately downstream of nucleotides 1833 contained elements that in some manner enhanced binding. Folding of the L25 binding site presumably is an autonomous event that is independent of any trans- /

cis- acting factors located on the periphery of the binding site. It is suggested that the 5' distal fragments might have an indirect influence on the r-protein binding, such that the folding of the 5' proximal fragment permits the protein to bind. However it has yet to be determined what auxillary sequences, if any, are neccessary to mediate correct folding of the binding site [El-Baradi et al,1987].

The overall interaction of L25 is within the A site of the peptidyl transferase center of the 50S / 60S ribosomes [Garrett,1971]. The probable function of L23 may be in mediating proper positioning of the 3' termini of the amino acyl and the peptidyl tRNAs. This site is not only structurally but functionally conserved among prokaryotes and eukaryotes [Garrett,1971].

The mouse equivalent of the L25 binding site [refer to fig.7], though structurally similar to its yeast counterpart, is unable to bind the yeast L25 protein. Several plausible explanations exist. The larger eukaryotic specific variable V9 region in mouse could have a deleterious effect on L25 binding. Since the eukaryotic specific hairpin is absent in the 23S rRNA, it may not have an active role in binding. However the question remains why in some species [insects & protozoans] a breakage occurs within this V9 region [refer to fig.6].

An amazing observation was made, that even though EL23 / YL25 demonstrate extensive conservation of primary / secondary structure , there is limited homology between the two proteins. This homology is restricted to the carboxy terminus of the proteins [16 amino acids (a.a.) out of 42

a.a. can be aligned, refer to fig.10]. The two proteins demonstrate amino acid homology at only three small and widely dispersed sites. In fact EL23 is only 28% homologous to BL23 from *B. stearothermophilus* [Kimura et al 1985]. Therefore, it is tempting to assume that these three homologous stretches might play a significant role in the interaction with lr RNA or functioning of the ribosome. However, due to the large size and structural complexity of the lr RNA binding site, it seems unlikely that the lr RNA interaction would be solely dependent upon such a small stretch of amino acids [i.e. KKA(*)V(*)L conserved motif].

Although several ribosomal proteins have been isolated from the ciliated protozoan *T.thermophila* [Petridou et al 1984], no L25 - like homologue has been identified as yet [see fig.11]. Heterologous

binding experiments have revealed that a *T.thermophila* T7 precursor rRNA transcript is unable to bind yeast YL25 [Ware,submitted]. When chimeric 26S rRNAs in which the variable V9 regions from yeast were replaced with V9 sequences from mouse and *T.thermophila* were used in binding studies, no significant change in binding efficency or polysomal distribution of 60S subunits was observed [W.Musters,personal communication]. This suggests that the binding requirements in gap processing and non-processing organisms may be quite complex.

Conserved Domain at the C-terminus of L25 Homologues.

Yeast	Lys-Lys-Ala-Tyr-Val-
E.coli	Lys-Lys-Ala-Tyr-Val-

Fig. 8 : AMINO ACID SEQUENCE HOMOLOGIES BETWEEN YEAST L25 [S.c.] AND *E.coli* [E.c.]. Boxed in sequences represent identical amino acid stretches. [taken from Raue et al. 1989]

MOLECULAR WEIGHTS OF RATLIVER RIBOSOMAL PROTEINS

40S		60S	
S1	44	L1	38.6
S2	41	L2	32.4
S3	38.1	L3	53
S4	35.3	L4	53.7
S5	29.8	L5	45.8
S6	38.5	L6	48.5
S7	31.3	L7	38.3
S8	32.5	L8	35.8
S9	27.2	L9	31.8
S10	27.3	L10	34.8
S11	26.3	L11	26.5
S12	38.5	L12	23
S13	21.2	L13	33.9
S14	24.9	L14	32.3
S15	25/3	L15	30
S16	20.5	L16	24.8
S17	22.5	L17	29.5
S18	21.5	L18	29
S19	20.5	L19	32
S20	10.1	L20	27
S21	18.8	L21	28
S22	—	L22	—
S23	23.9	L23	23.3
S24	22.6	L24	24
S25	22.1	L25	23.7
S26	19.1	L26	25.6
S27	16.9	L27	21.9
S28	11.5	L28	22.7
S29	10	L29	24
S30	12.4	L30	21.7
S31	25.3	L31	20.8
		L32	20.7
		L33	22
		L34	14.5
		L35	19.7

Fig.11 : COMPARISON OF RIBOSOMAL PROTEINS OF *Tetrahymena thermophila*. [taken from Petridou et al. 1984]

I propose to isolate an L25 homologue from *T.thermophila* in order to study its interaction with precursor and mature 26S rRNA. If differences existbetween gap processing organisms [*T.thermophila*] and organisms that do not [yeast], it may help to understand the significance of the gap region. It is quite possible that more than one L25 homologue[s] exist in gap processing organisms. It is also possible that gap processing may be a prerequiste for L25 binding. Removal of nucleotides from this eukaryotic specific variable V9 region may facilitate L25 binding.

CHAPTER II

MATERIALS & METHODS

[1] Strains

Yeast *Saccharyomyces cerevisieae* [ATCC] were grown at 25 ° C in yeast nutrient media [1% Bacto yeast extract , 2 % Bacto peptone , 2% sucrose] for 16 hrs with mild shaking. *T.thermophila* cells [ATCC 30384] were grown in ATCC medium 357 at 25 ° C for 24 hrs with mild shaking to 3 x 10^5 cells / ml concentration; cells were induced to over-synthesize ribosomal proteins by starvation in minimal media [5mM Tris-HCl pH 7.3, 47mM NaCl, 1mM MgSO4] for 17 hrs at 25°C and then

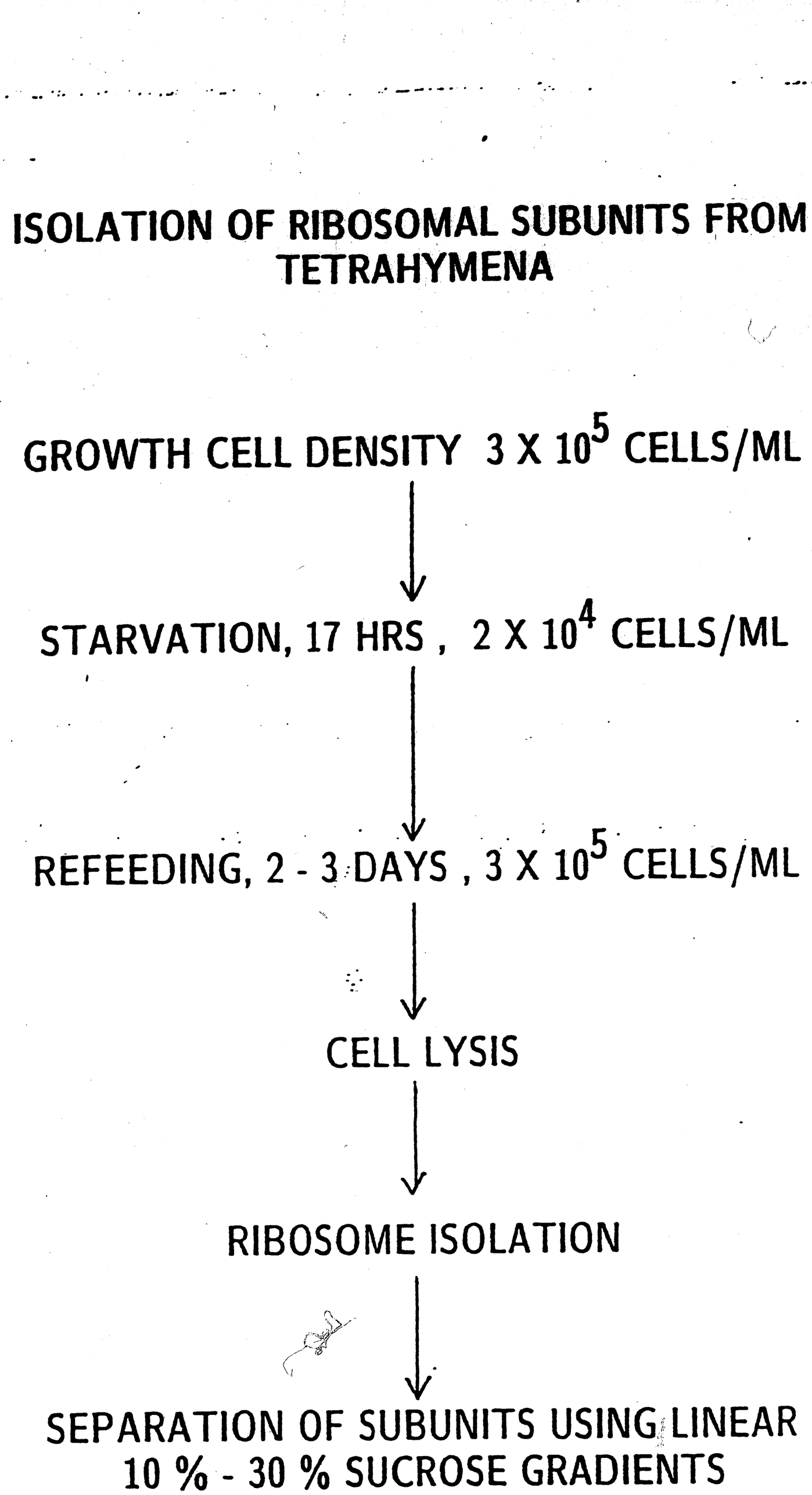

Fig. 9 : FLOW DIAGRAM FOR THE ISOLATION OF RIBOSOMAL SUBUNITS FROM *T.thermophila*. [Cuny et al,1982]

cells were refed in nutrient media to a cell density of 2 - 3 x 10^5 cells / ml [see fig.12].

Radio-labelled yeast and *T.thermophila* cells were prepared in the following manner: *Tetrahymena* cells were grown at 25° C for 16 hrs before transferring to ATCC medium 357 containing ^{32}P - labelled H_3PO4 [ICN, 1.0 mCi / 100 ml media] and allowed to grow for an additional 24 hrs at 25° C. Yeast cells were labelled in a similar manner.

[2] Isolation of ribosomes

T.thermophila cells were starved and refed as described in methods above and ribosomes were isolated according to Petridou et al,1983. Cells were harvested at 2000 x *g* [Spinco rotor JA14 10,000 rpm] for 45 mins 4° C. The cell pellet was frozen at

- 70° C, thawed on ice, and resuspended in RS buffer [0.01 M NaCl, 0.015 M $MgCl_2$, 0.01 M Tris pH 7.5], homogenized in a Dounce homogenizer with 1 % [w/v] Triton X-100 and 0.5 % [w/v] sodium deoxycholate. Cellular debris was eliminated by centrifugation at 15,000 x *g* [Spinco rotor JA20, 10,000 rpm] for 30 mins 4° C. The supernatant was re-centrifugated with the protease inhibitor iodoacetamide [final concentration of 5 mM from a 100 mM stock solution] at 100,000 x *g* [Beckman rotor SW41, 24,000 rpm] for 90 mins at 4° C to obtain the crude ribosome pellet. The crude ribosomal pellet was resuspended in High Salt buffer [0.5 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris pH 7.5] and further purified by centrifugation at 150,000 x *g* [Beckman rotor SW41, 30,000 rpm]. The ribosomal pellet was resuspended in Dissociation Buffer [20 mM Tris-HCl pH 7.4, 16 mM $MgCl_2$, 1 M KCl,

12 mM β - ME, 0.2 M EDTA] and stored at -70°C.

Ribosmes were dissociated into subunits by centrifugation through a 10 % - 30 % linear sucrose gradient in dissociation buffer [Beckman SW27, rotor] at 20,000 rpms for 17 hrs at 4° C. One ml fractions were collected and absorbance at 260 nm was monitored. Fractions containing the 60 S and 40 S peaks were pooled and centrifuged at 80,000 x *g* [Beckman rotor SW41 25,000 rpm] for 20 hrs at 4° C. Subunit pellets were resuspended in standard dialysis buffer [10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 50 mM KCl, 6 mM β - ME] and stored at -70° C.

Yeast ribosomes were prepared according to methods described by El-Baradi et al,[1984]. Cells were harvested at 2000 x *g* [Spinco rotor JA14, 10,000 rpm] for 45 mins at 4° C. The cell pellet was

frozen at - 70° C, thawed on ice in 2 mls cold Buffer A [10 mM Tris-HCl pH 7.5 , 5 mM Mg Acetate , 10 mM β - ME , 10 mM KCl] and lysis was carried out with glass beads. The cell lysate was filtered with cold Buffer A and cell debris was removed by centrifugation at 8000 x *g* [Spinco rotor JA20, 13,000 rpm] for 20 mins at 4° C. The supernatant was re-centrifuged at 105,000 x g [Beckman rotor SW41, 30,000 rpm] for 90 mins at 4° C. The crude ribosomal pellet was resuspended in Buffer D [50 mM Tris-HCl pH 7.7, 12 mM Mg Acetate, 20 mM β - ME, 0.8 M KCl]. Ribosomal subunits were separated on 10 % - 30 % linear sucrose gradients and concentrated as described for Tetrahymena cells.

[3] Isolation of core ribosomal proteins

[a] *T.thermopjhila* core proteins

T.thermophila core ribosomal proteins were prepared as per yeast LiCl extraction methods [El-Baradi et al., 1984]. *T.thermophila* 60S subunits [~ 2000 - 5000 Ab 260nm] were dialysed against standard dialysis buffer containing 1.8 M LiCl for 3 hrs at 4° C. LiCl extracted proteins were pelleted by centrifugation at 12,000 rpm in a Fisher Scientific microfuge for 30 mins at 4° C. The supernatant contains 26S rRNA bound with core ribosomal proteins. The RNA was removed by acetic acid precipitation. One tenth volume of ice cold 1 M $MgCl_2$ and 2x volume ice cold 65 % glacial acetic were added in rapid succession, the mixture stirred at 4° C for 45 mins, and the RNA was pelleted by centrifugation at 13,000 rpm for 15

mins in a Fisher Scientific microfuge. The pellet was washed briefly with 65 % glacial acetic acid. The combined supernatant was dialysed against 5 % glacial acetic acid, lyophilized and stored at - 70° C [see fig.13].

[b] Yeast L25 protein

Yeast L25 ribosomal protein was prepared as per El-Baradi et al.[1984], with some minor modifications. 60 S subunits [~ 2000-5000 Ab 260 nm] were dialysed against standard dialysis Buffer with 1.8 M LiCl for 4 hrs at 4°C.

[4] Preparation of *in vitro* transcripts

In vitro transcripts were prepared and radio-labelled using the RiboProbe TM Transcription system

[Protocol # 1] from Promega Biotec. Transcripts were labelled with α - ^{32}P- CTP obtained from ICN.

T.thermophila precursor T7 transcript [pGB500] was prepared from pGBTetBH500 [V.C. Ware, unpublished results]. This plasmid contains the 500 bp BgII/HindIII fragment isolated from plasmid pRP9 containing the gap region from *T.thermophila* cloned into the BamHI / HindIII sites of pGEM3-Blue [Promega]. A run off transcript was prepared by cutting the plasmid with HindIII and using 50 μCi α - ^{32}P - CTP in water [ICN] and T7 polymerase [Promega]. The DNA template was removed with RQ1 TM DNase [Promega] for 15 mins at 37° C. The transcription mixture was extracted with phenol:chloroform [1:1] followed by chloroform alone, and the RNA transcript was resuspended in 10 μL sterile distilled water.

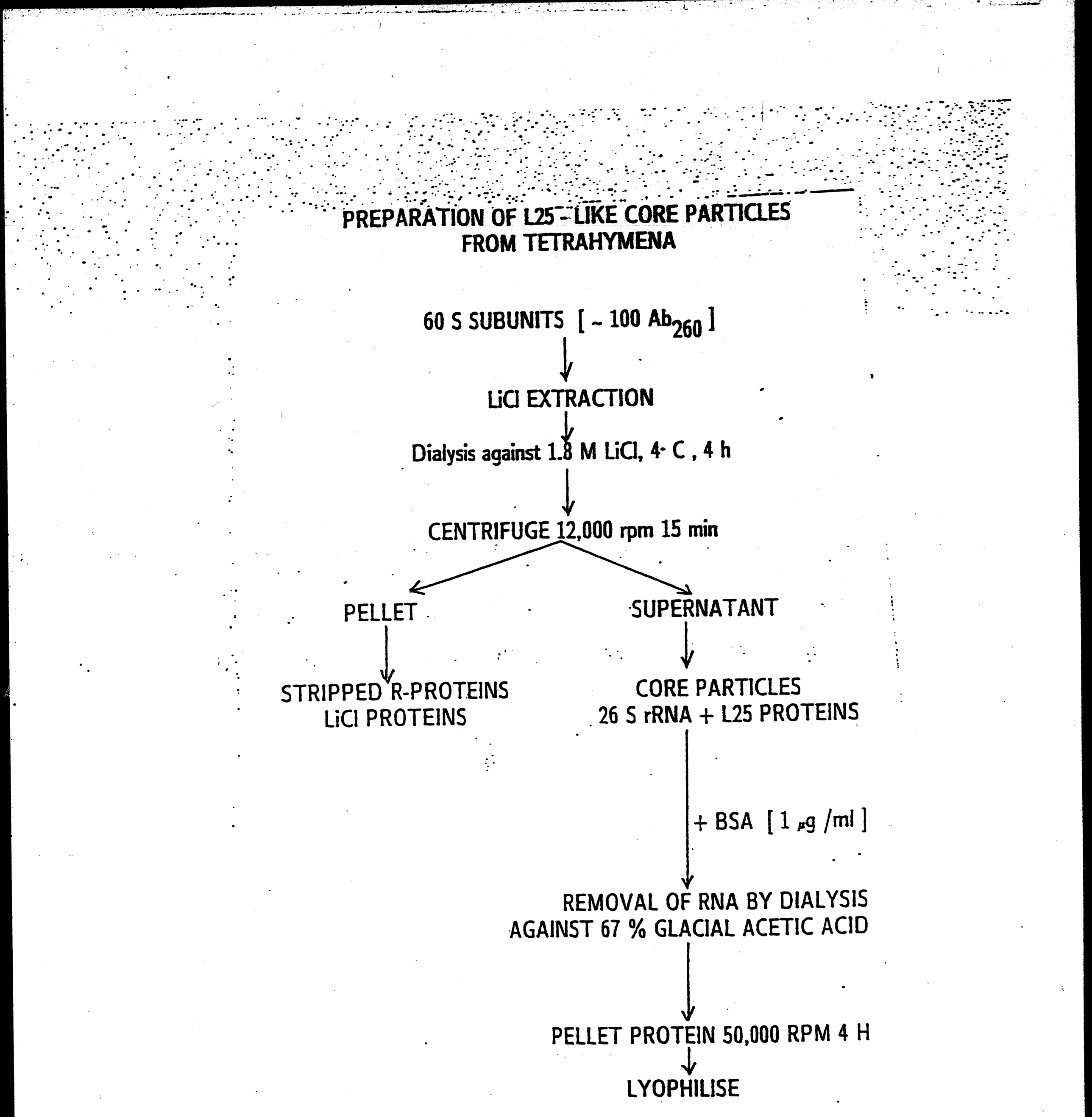

Fig. 10 : FLOW DIAGRAM FOR THE PREPARATION OF L25 CORE PARTICLES FROM *T.thermophila*. [ref. El-Baradi et al. 1985]

Yeast transcript was prepared from plasmid [pSY26XH] containing the entire L25 binding site [a kind gift from H.Raue]. pSY26XH was cut with AccI and run off transcripts were produced using SP6 polymerase [Promega] and α - ^{32}P - CTP [ICN], in a similar method as described for *T.thermophila*.

[5] Isolation of 26S rRNA

T.thermophila 60S and yeast 60S ribosomal subunits labelled with ^{32}P were isolated as described in methods 1 & 2. Subunits were stripped of their proteins by phenol:chloroform [1:1] extraction, and the 26S rRNA was resuspended in 10 μL of sterile water and stored at - 70°C.

[6] SDS - polyacrylamide electrophoresis of ribosomal proteins

Isolated core ribosomal proteins were identified by SDS-PAGE. Total ribosomal proteins from *T.thermophila* and yeast were prepared by TCA precipitation. Subunits were precipitated with 100 % [w/v] TCA [final concentration 10%], incubated at 0° - 4° C for 30 mins, and centrifuged in a microfuge at 4° C for 30 mins. The protein pellet was washed several times with 95 % ethanol:ether [1:1], vacuum dried and resuspended in 1 X sample buffer.

Total ribosomal proteins, LiCl extracted proteins and molecular weight markers [Sigma] were electrophoresed on 12.5 % - 20 % linear gradient SDS gels for 3 - 4 hrs at 20 mA constant current. Gels were stained with Coomassie Blue and dried on a gel

dryer.

T.thermophila core proteins were electrophoresed on 12.5 % - 20 % linear gradient SDS-gels and stained with silver reagent [Bio-Rad silver stain kit].

[7] Nitrocellulose Filter Binding Assay

Nitrocellulose filter binding assay [NFB] was executed according to yeast protocols [El-Baradi et al ,1984]. Equivalent amounts of ^{32}P-labelled rRNA [i.e. 26S rRNA or SP6 / T7 *in vitro* transcripts] were incubated with increasing amounts of either *T.thermophila* core proteins or yeast L25 protein in binding buffer [20 mM Tris HCl pH 7.4, 20 mM $MgCl_2$, 300 mM KCl, 6 mM β - ME], for 15 mins at 37° C. Incubation mixtures were then filtered through nitrocellulose membrane filters [Schleicher &

Schuell grade BA85, 24 mm diam., 0.45 μ], washed three times with 0.5 mls Binding buffer and the protein protected radiolabelled RNA fragments retained on the filters were estimated by liquid scintillation counting [see fig.14] .

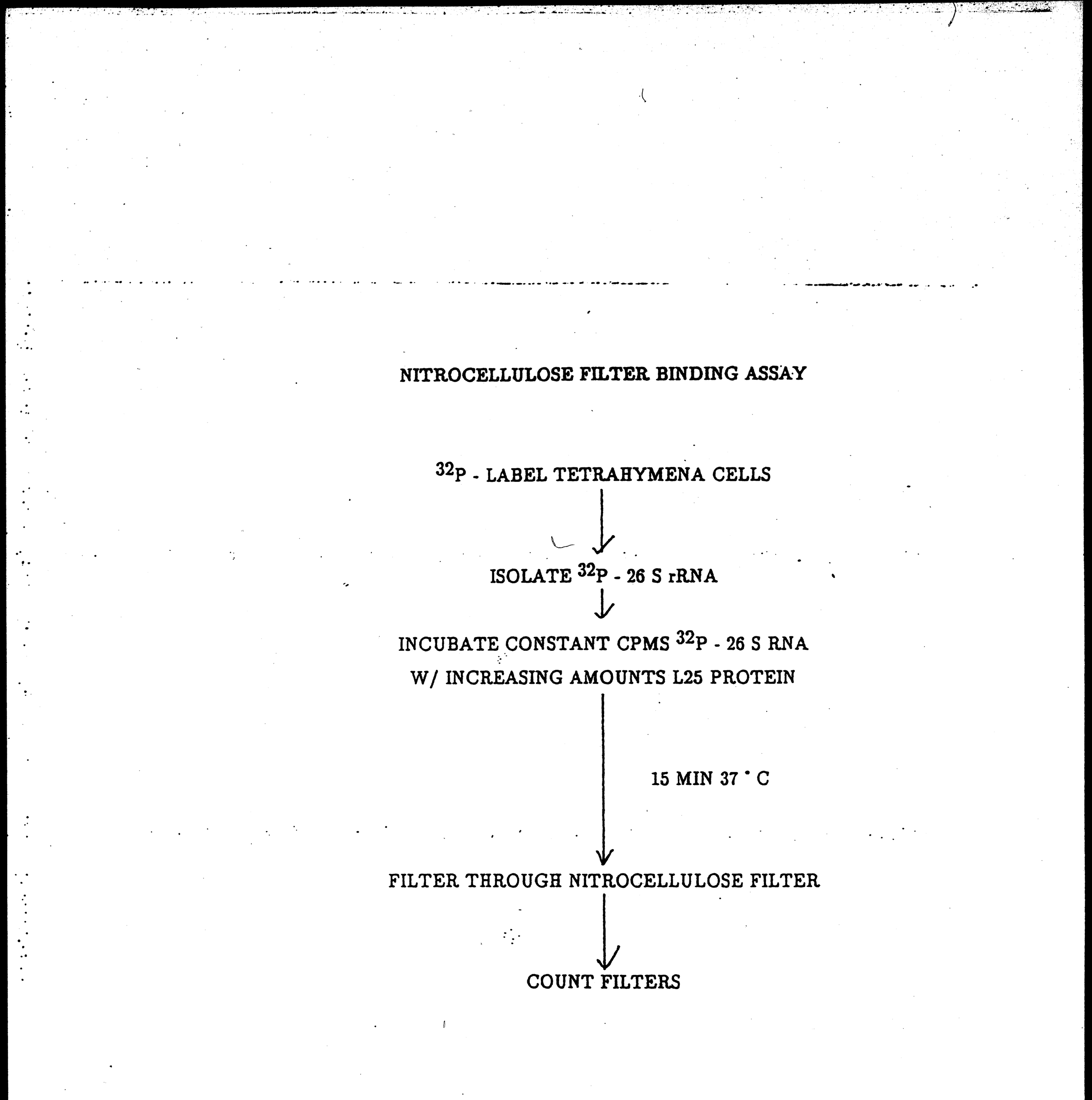

Fig. 11 : SCHEMATIC REPRESENTATION FOR NITROCELLULOSE FILTER BINDING ASSAY.

[8] RNA polyacrylamide gels

Native RNA - protein polyacrylamide gels [RNP gels] were run according to Branlant et al [1973]. RNA-protein complexes were fractionated on a 8 % polyacrylamide gel [11 cm x 24 cm x 0.4 mm] made up in TMA buffer [0.01 M Mg acetate, 0.005 M Tris-acetate pH 8] or TBE buffer [0.05 M Tris, 0.01 M EDTA, 0.05 M Boric acid pH 8.3]. Prior to sample loading, gels were pre-run at 30 mA for 30 mins. Samples were prepared in a 1 X sample buffer [50 % (w/v) sucrose with 1 % (w/v) bromophenol blue] or 10 X sample buffer [0.5 M Tris, 0.5 M Boric acid, 100 mM EDTA pH 8.3, 50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.25 % (v/v) xylene cyanol]. Electrophoresis was carried out for 3 - 4 hrs at 30 mA at a potential difference of 100 - 200 V. The xylene cyanol was allowed to run off while the

bromophenol blue reached the bottom of the gel. Gels were autoradiographed.

Denaturing RNA sequencing gels were prepared according to Maniatis et al [1982]. 8 % or 10 % polyacrylamide 7 M urea gels were run in 1 x PB buffer at 20 mA for 3 - 4 hrs and autoradiographed.

RNA fragments were extracted from native or denaturing gels using the crush & soak method [Maniatis et al.1982]. RNA fragments were excised and soaked in SAE buffer [0.1 % SDS, 0.5 M ammonium acetate, 0.1 M EDTA] for 12 hrs at 37° C, briefly centrifuged at 12,000 rpms in a microfuge at 4° C and passed through a disposable syringe packed with silica glass wool and 3M Whatman filter paper, to filter out polyacrylamide bits. The supernatant was ethanol precipitated, washed several times with 70 %

ethanol and resuspended in sterile water.

[9] RNase T1 Protection Assay

RNase T1 protection experiments were carried out as described by El-Baradi et al [1985], Branlant et al [1983], Garrett et al [1971]. ^{32}P-labelled rRNA [i.e. 26S rRNA or SP6 / T7 transcripts, 10^4 - 10^5 cpms / 5 μg RNA] together with 40 ng unlabelled 17S rRNA were incubated with saturating amounts of *T.thermophila* core proteins or yeast L25 protein at 37° C for 15 mins in binding buffer. Protein - RNA complexes were partially digested with RNase T1 [10 units / 5 ng RNA] in T1 buffer [10 mM Tris-HCl pH 7.4, 50 mM KCl, 6 mM β - ME, 10 mM $MgCl_2$] at 0° C for 30 mins. Hydrolysates were directly loaded on to 10 % Native RNP gels and electrophoresised for 3 - 4 hrs at 20 V/cm [20mA]. Gels were visualized by

autoradiography. Bands containing the RNP were excised , dialysed against 8 M urea for 2 hrs at room temperature, to remove the electrophoresis buffer and facilitate dissociation of RNA and protein. The gel fragment was then loaded on a 10 % polyacrylamide 7 M urea sequencing gel and autoradiographed [see fig.15] .

IDENTIFICATION OF L25 BINDING SITE ON 26S rRNA

RNASE T1 PROTECTION ASSAY

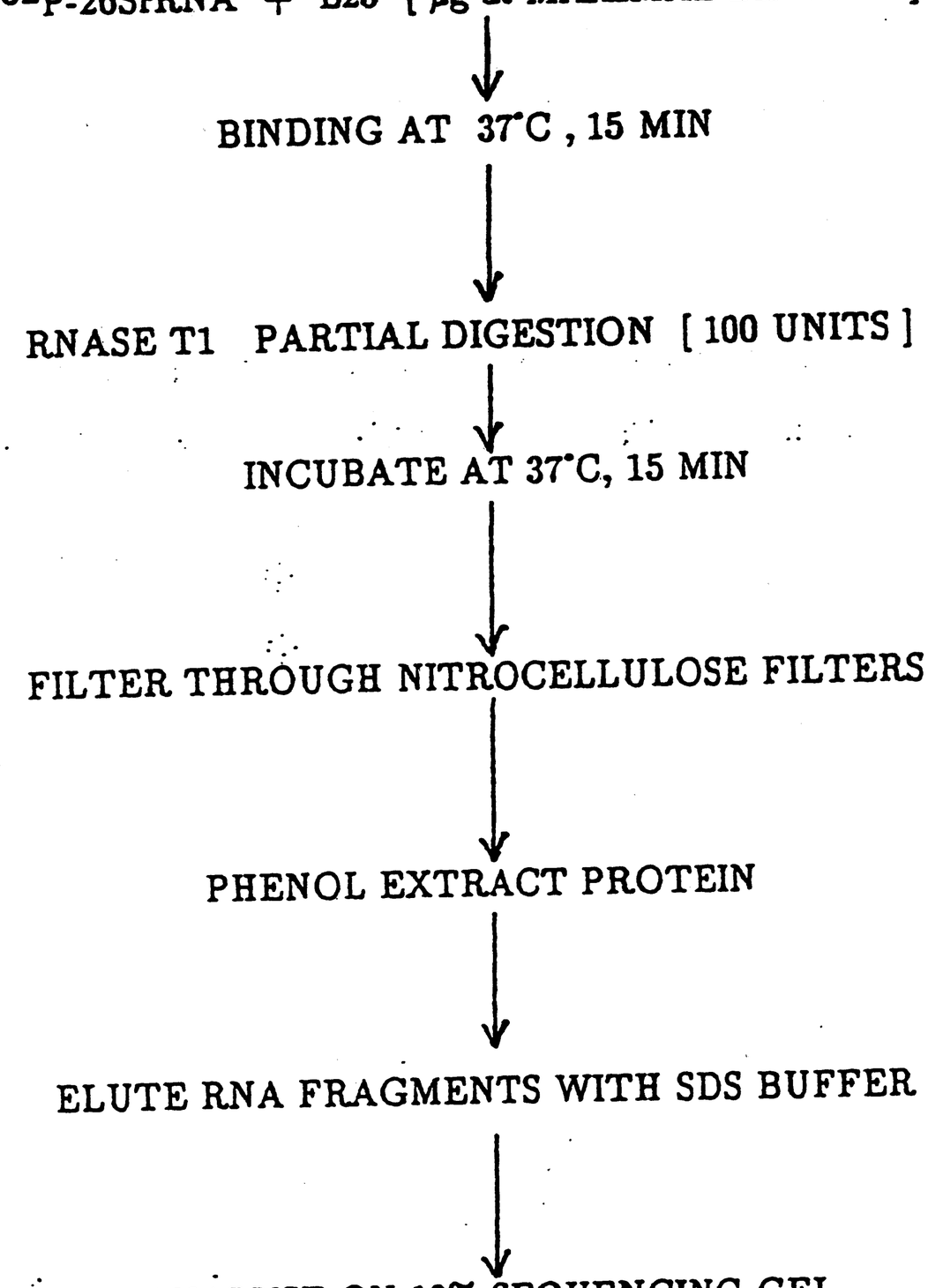

Fig. 12 : SCHEMATIC REPRESENTATION OF RNase T1 PROTECTION ASSAY

CHAPTER III

RESULTS & DISCUSSIONS

SECTION I

RESULTS

In order to investigate the existence of an L25 homologue in the ciliated protozoan *T.thermophila* , ribosomal core particles containing a complex of RNA and protein were isolated. The ability of these ribosomal proteins to bind specifically to the 26S rRNA was examined using a Nitrocellulose Filter Binding Assay. In order to determine if an L25 candidate existed among the core proteins Rnase T1

protection experiments were performed.

BIOCHEMICAL ISOLATION OF CORE PROTEINS FROM YEAST AND T.thermophila

The existence of a putative L25 homologue in *T.thermophila* was investigated using LiCl extraction methods previously successful for yeast, [see Chapter II for extraction details]. Treatment of yeast and tetrahymena 60 S ribosomal subunits with increasing concentrations of LiCl results in methodical stripping of certain ribosomal proteins from the subunit while a core particle consisting of RNA and a few early binding proteins remains [El-Baradi et a 984]. Comparison of LiCl extracted 60S

ribosomal proteins with total 60S ribosomal proteins, revealed that a few protein were not present in the LiCl extracted lane [2] but were present in the toatl 60S ribosomal protein lane[3], [see fig.16 lanes 2 & 3]. The early binding proteins retained on the core particle after treatment with LiCl were identified to be approximately 20 Kd in size [data not shown]. A similar electrophoretic pattern was observed in *T.thermophila* 60 S subunits. LiCl extracted 60S ribosomal proteins were compared with total 60S ribosomal proteins from *T.thermophila*. A group of 15 Kd proteins that were not present in the LiCl extracted lane but were present in the total

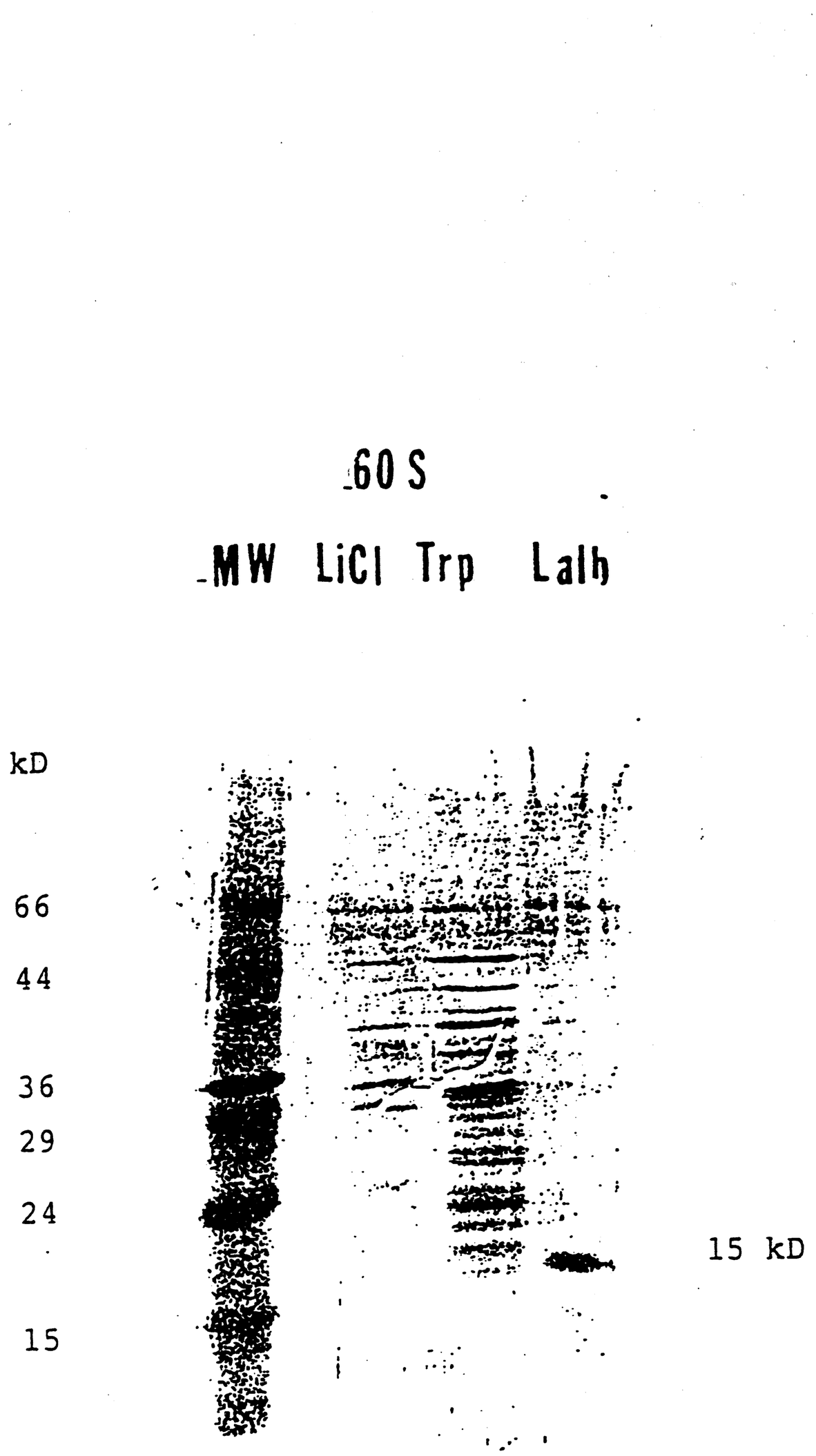

Fig. 13 : 12.5 - 20 % LINEAR GRADIENT SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF YEAST TOTAL 60S RIBOSOMAL PROTEINS. Lanes; MW : Molecular weight markers; LiCl: LiCl extracted 60S ribosomal proteins; Trp: total 60S ribosomal proteins and Lalb: 15 Kd molecular weight marker Lactalbumin.

60S lane was observed [see fig.17, lanes 3 & 4]. Total ribosomal protein pattern was very similar to that obtained by Petridou et al 1984. The core ribosomal proteins were further identified by gel electrophoresis. The core particle appeared to consist of at least three ribosomal proteins of approximately 15 Kd in size see fig.18,lane 2.

Clearly some proteins present in the total 60S protein lanes are absent from the LiCl stripped proteins. Presumably, these proteins are retained on the 60S subunit even after LiCl extraction and would constitute core proteins.

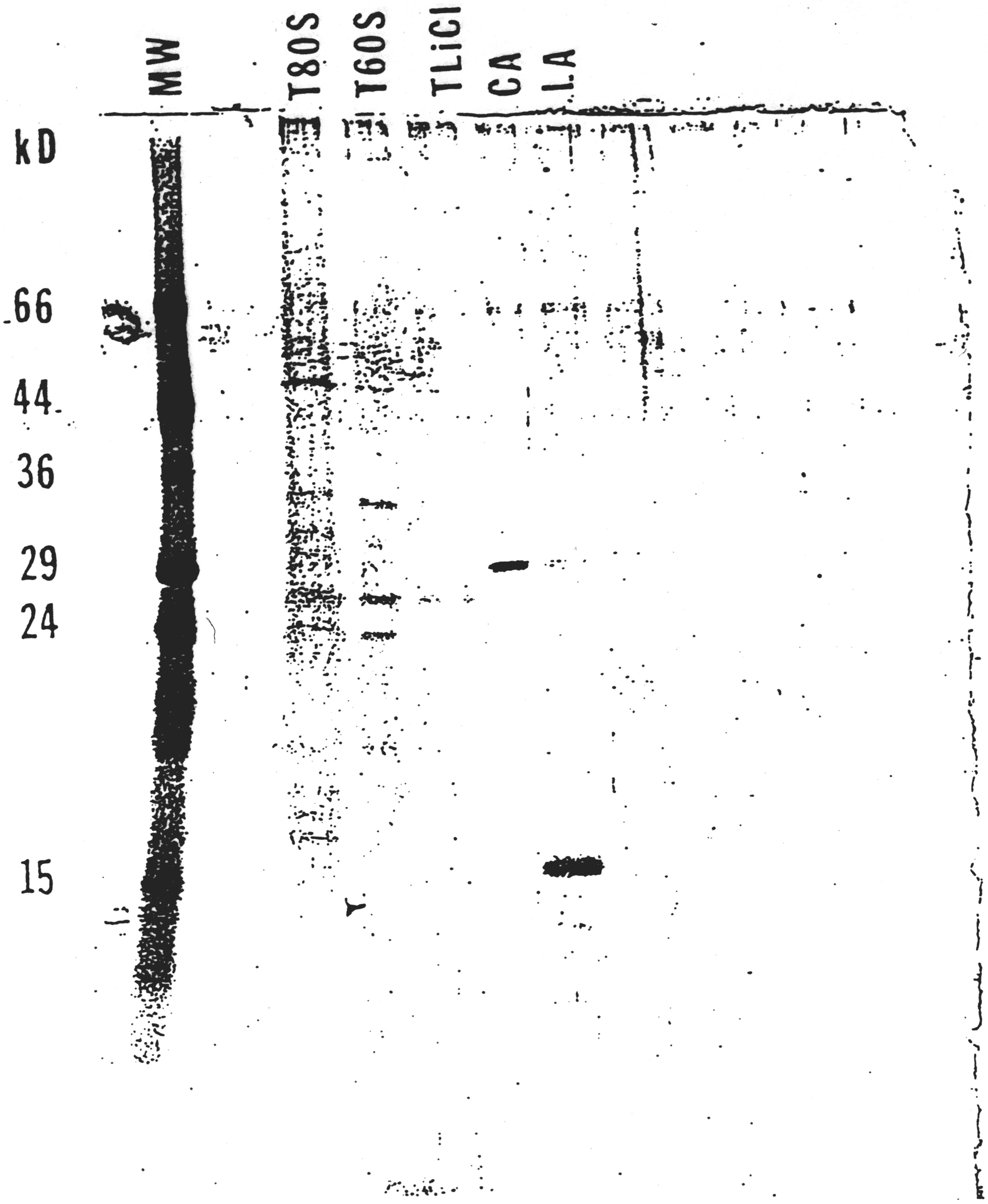

Fig. 14 : 12.5 - 20 % LINEAR GRADIENT SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF *T.thermophila* RIBOSOMAL PROTEINS . Lanes; MW: Molecular weight markers ; T80S: total 80 S ribosomal proteins ; T60S: total 60 S ribosomal proteins; TLiCl: LiCl extracted 60S ribosomal proteins ; CA & LA: molecular weight markers carbonic anhydrase [29 Kd] and Lactalbumin [15 Kd],respectively.

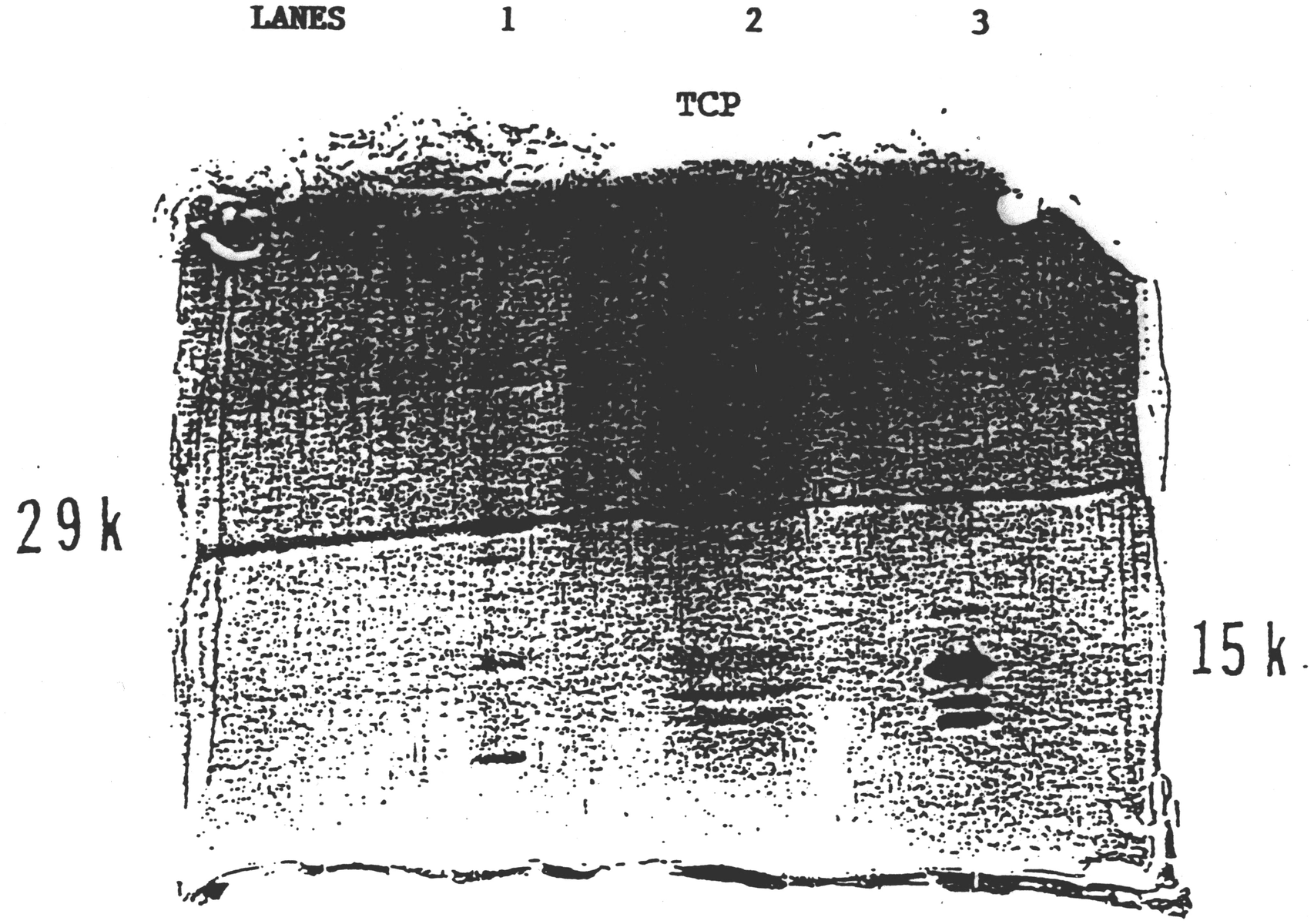

Fig. 15 : 12.5 - 20 % LINEAR GRADIENT SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF ISOLATED *T.thermophila* CORE RIBOSOMAL PROTEINS. Lanes 1 & 3 : Molecular weight markers carbonic anhydrase [29 Kd] lane 1 and lane 3 lactalbumin [15 Kd]. Lane 2 represents the isolated core ribosomal proteins from tetrahymena using LiCl extraction methods. Proteins were visualised by Silver staining . Concentration of protein per band was 50 ng.

BINDING OF CORE PROTEINS TO RNA

Based on the LiCl extraction of 60S subunits, it was determined that three proteins remain on the *T.thermophila* core particle. The ability of these presumed RNA-binding proteins to interact with RNA was confirmed using filter binding assays. Binding studies were performed with both yeast and Tetrahymena core proteins.

Yeast total rRNA demonstrates a saturating curve with increasing amounts of YL25 ribosomal protein [see fig.19]. At saturating levels of protein, 15 % the RNA was retained on the filter. A similar binding trend was observed for Tetrahymena total rRNAs. Saturation occurs at concentrations exceeding 1000 f moles [see fig.19 B]. These binding

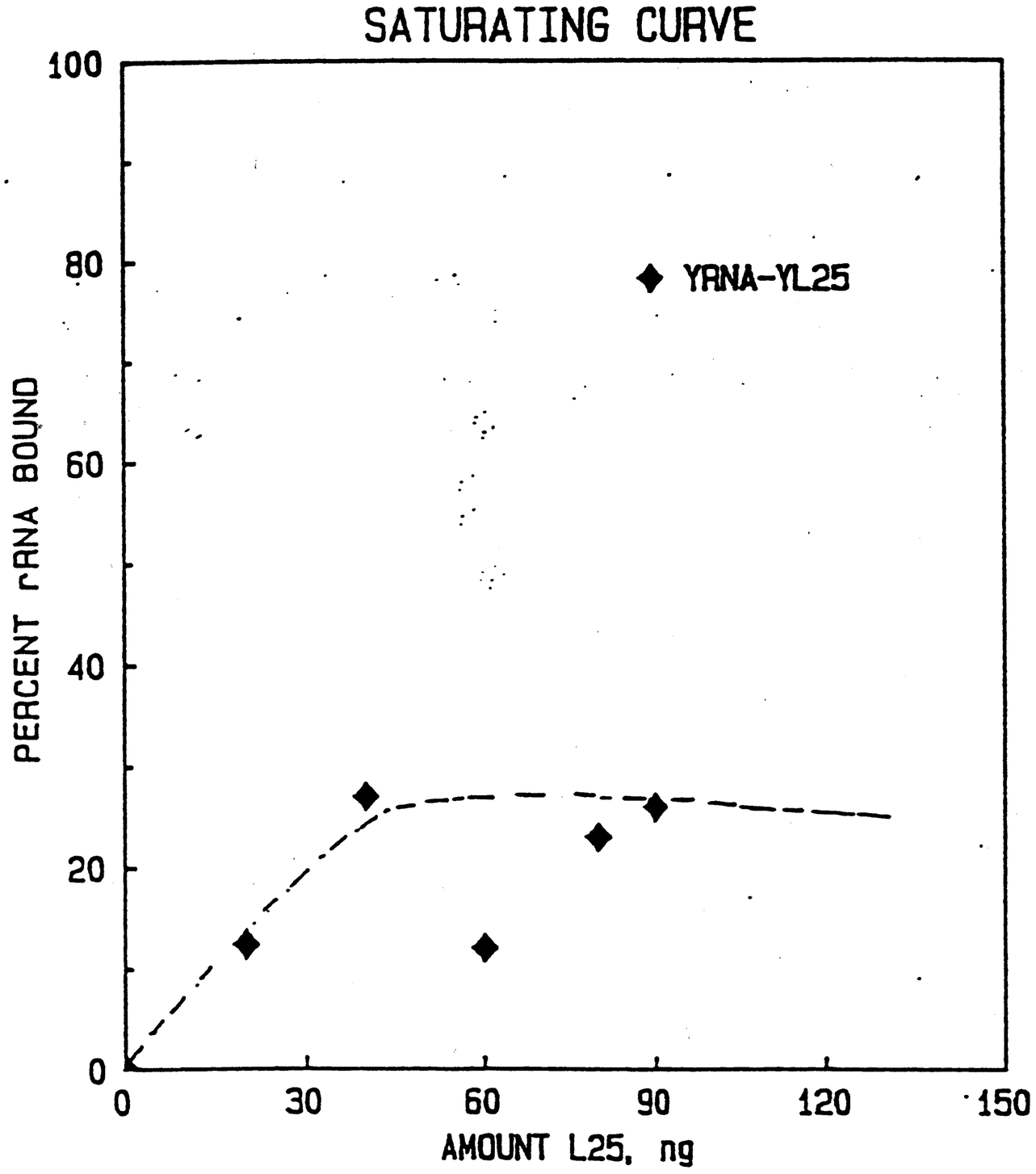

Fig. 16 : [A] SATURATION CURVE OF THE INTERACTION OF YEAST TOTAL rRNA WITH YEAST L25 RIBOSOMAL PROTEIN . A constant molar concentration of ^{32}P - labelled yeast total rRNA [20 ng ; 1500 cpm / data point] was incubated with increasing amounts of YL25.

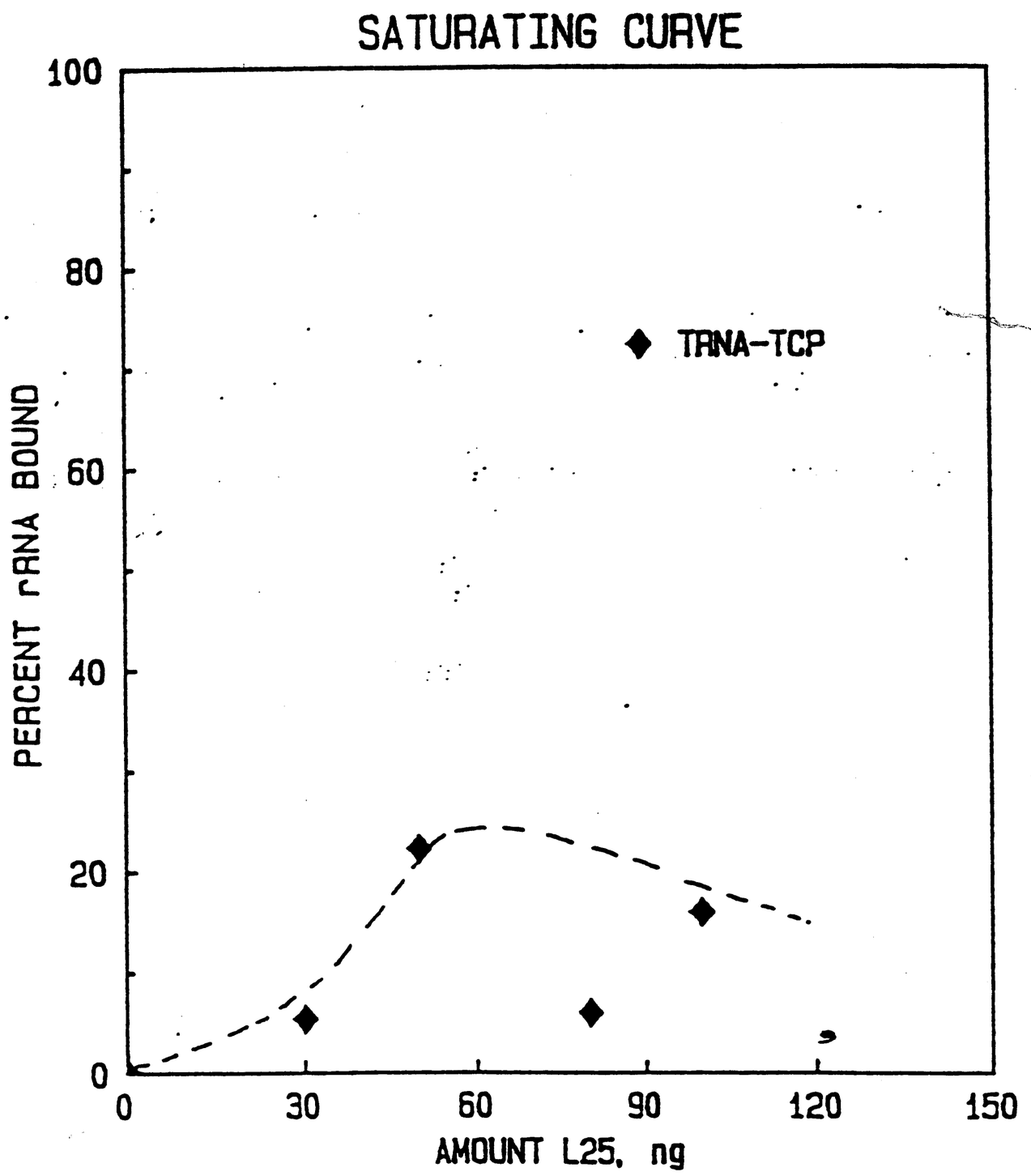

Fig.17 : [B] SATURATING BINDING CURVE FOR THE INTERACTION OF *T.thermophila* TOTAL rRNA WITH CORE RIBOSOMAL PROTEINS. A constant amount [20 ng / 1500 cpm / data point] of ^{32}P - labelled *T.thermophila* total rRNA was incubated with increasing amounts of tetrahyemna core ribosomal proteins.

curves seem to indicate that the core proteins are capable of interacting with and binding to rRNAs.

The specificity of this binding interaction was tested with isolated 26S rRNAs, from both yeast and *T.thermophila*. In order to ascertain if these core proteins are specific for the 26S rRNA, binding interactions were compared between isolated 26S and 17S rRNAs. Both yeast and *T.thermophila* 17 S rRNAs were unable to bind core ribosomal proteins [see fig.20 A & B], indicating that the core proteins are specific for the large ribosomal subunit [see Fig.21 A & B].

The overall binding trend between *T.thermophila* and yeast 26S rRNAs did not seem to differ significantly. Twenty nanograms of yeast YL25 appears to be sufficent to saturate the binding interaction. Maximum amount of yeast 26S rRNA

retained on the filter was 8 % [Fig.21]. At saturation, 20 - 30 µg of T.*thermophila* TCP core protein appears to be sufficient to bind 22 % of 26S rRNA to the filter. At protein concentrations exceeding 30 µg a steady decrease in binding was observed [Fig.20 B]. Severe inhibition occurred at concentrations greater than 80 µg TCP protein.

Having established that the *T.thermophila* core proteins are specific for 26S rRNAs, it was of interest to ask if these proteins bind to mature and "precursor" substrates in a similar manner as determined by NFB assays.

One hypothesis states that the size of the variable V9 region may have a profound effect on L25 binding. Since the binding site for L25 is interrupted by the site where gap processing occurs

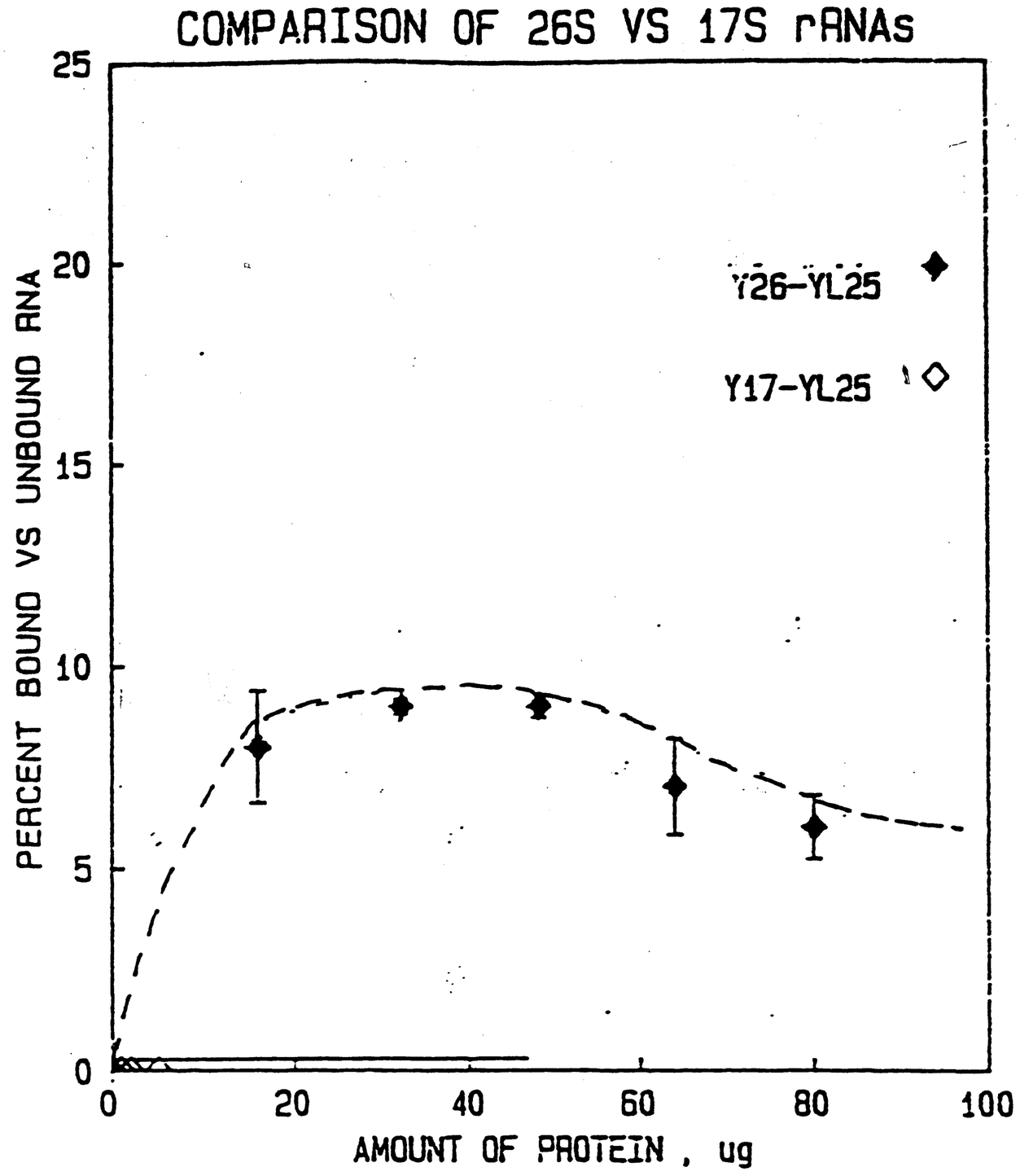

Fig. 18 : [A] COMPARATIVE ANALYSIS OF THE BINDING CURVES FOR YEAST 26 S AND 17 S rRNAs WITH YEAST YL25. Nitrocellulose filter binding assays were performed for ^{32}P- labelled yeast 26 S and 17 S rRNAs with yeast YL25 , as described previously for total rRNA binding curves. Constant amounts [~ 25 ng / 1000 cpm / data point] were incubated with increasing amounts in micrograms [μg] of yeast YL25 ribosomal protein. Percent of bound rRNA for each population [26S and 17 S rRNAs] were calculated. Each point represents a mean of 4 - 5 data points. The two curves were significantly different at a confidence level of > 99 %.

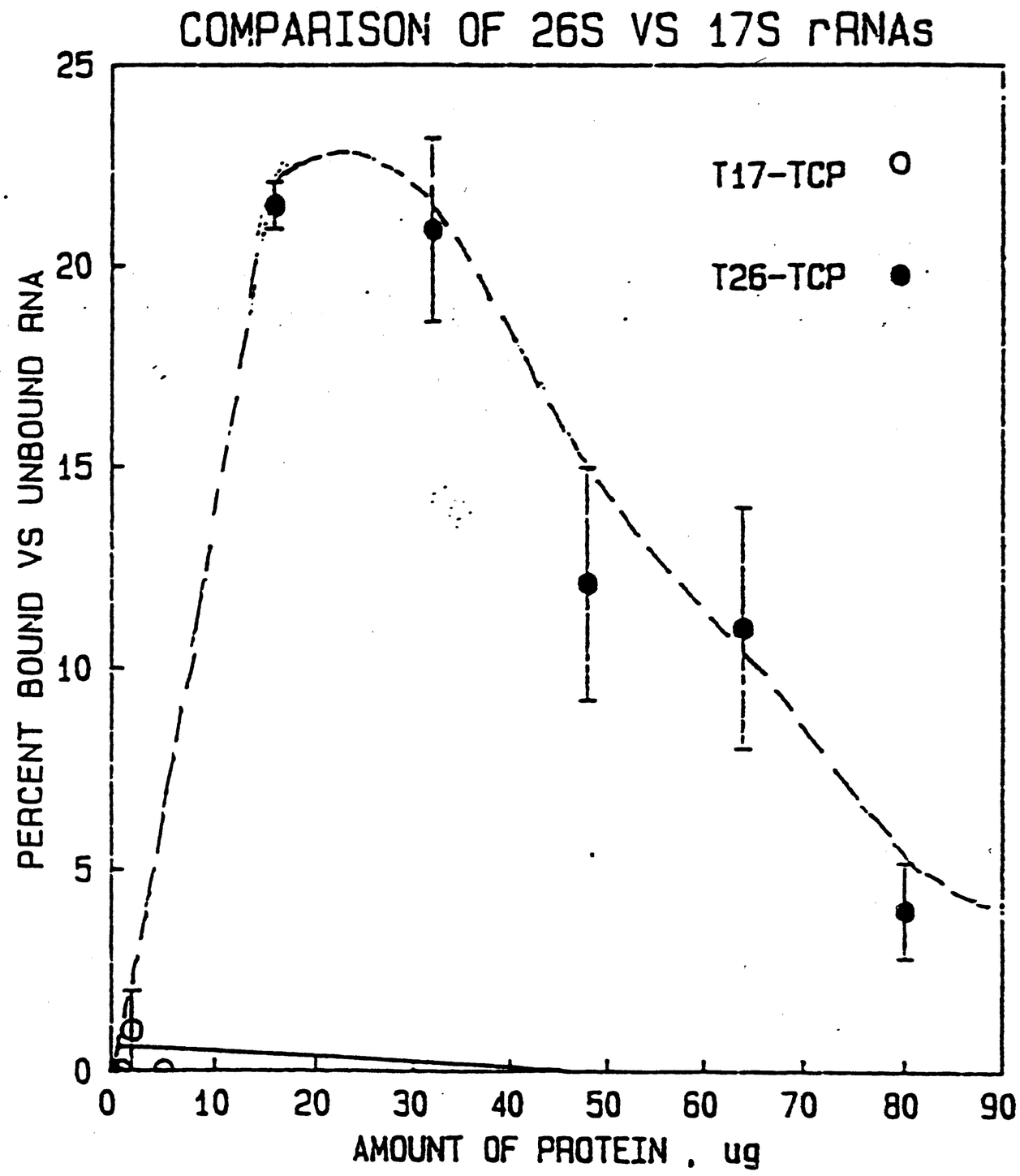

Fig. 18 : [B] ANALYSIS OF *T.thermophila* 26 S AND 17 S rRNAs BINDING. Nitrocellulose filter binding assays were performed with Tetrahymena ^{32}P - labelled 26 S and 17 S rRNAs and core ribosomal proteins [TCP].

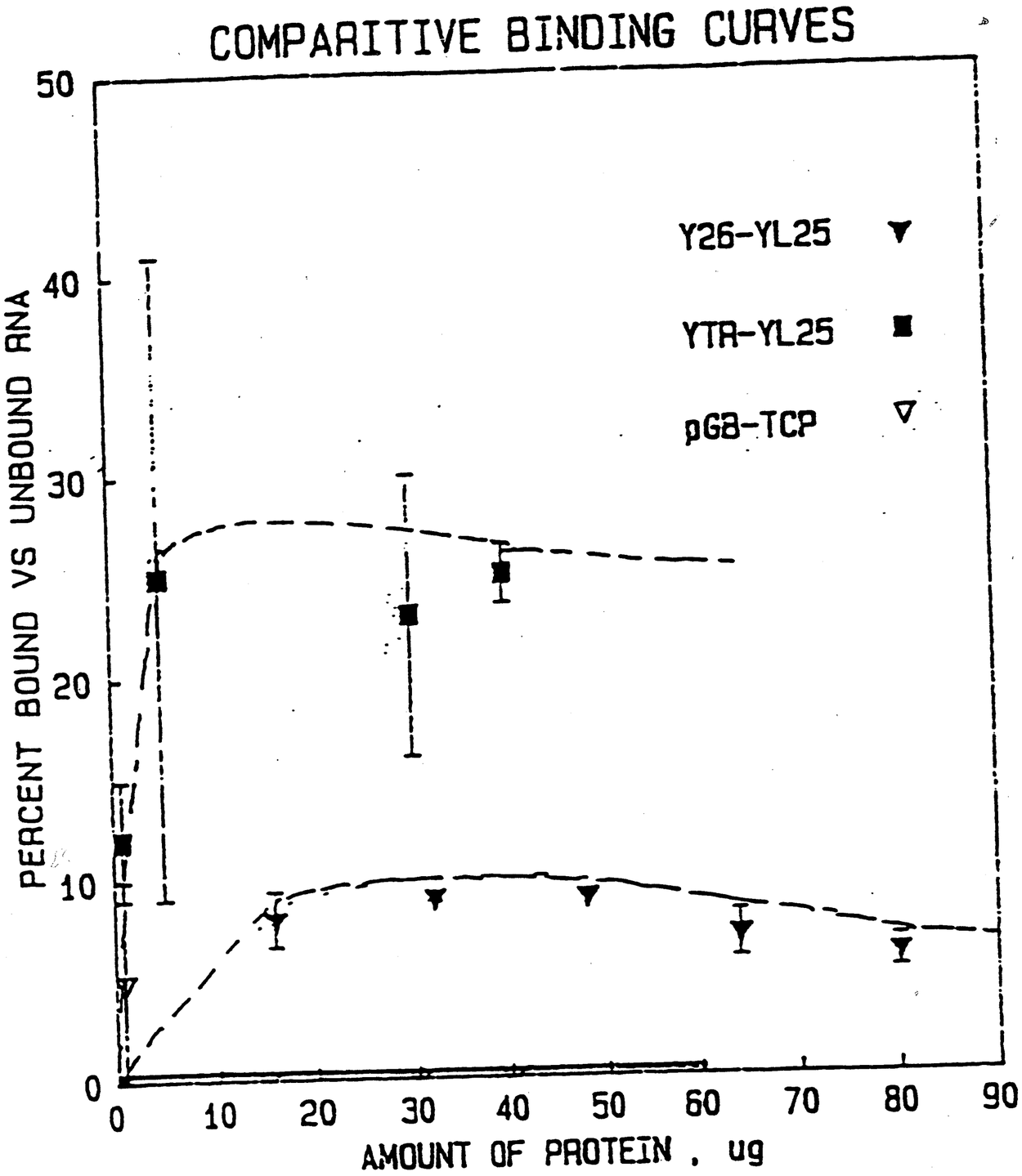

Fig.19 : L25 BINDING BETWEEN MATURE 26 S rRNA AND *in vitro* TRANSCRIPTS FROM YEAST AND *T.thermophila*. Nitrocellulose filter binding assay was performed as previously described in legend to Fig.19 with radiolabelled yeast 26 S rRNA ; yeast SP6 [YTR] and tetrahymena T7 [pGB] transcripts.

in ciliated protozoans [*T.thermophila*], processing at this site might facilitate L25 binding. In order to investigate this possibility, in vitro transcripts were prepared which contain sequences found in precursor rRNA, since *in vivo* unprocessed precursor pre-rRNAs are difficult to isolate in sufficent quantities.

An SP6 transcript [YTR] containing the entire L25 binding site in yeast as well as a T7 [pGB] transcript containing the presumptive L25 binding site in *T.thermophila*, were prepared as described in Materials & Methods. Filter binding assays were performed with yeast YTR and tetrahymena pGB transcripts. A 10 μg concentration of yeast YL25 ribosomal protein is sufficent to saturate binding of YTR rRNA transcript. Maximum amount of YTR transcript retained on the filters is 28 % [Fig.22].

At comparable protein concentrations, YTR transcript demonstrates a stronger binding interaction [28 %] than its mature counterpart 26S rRNA [8 %], in yeast. Tetrahymena core proteins did not retain the precursor pGB transcript on nitrocellulose filters. Percent RNA bound to filters was similar between Tetrahymena T26S [27%] and yeast transcript YTR [28%] [Fig.24 B]. However unlike yeast YTR, *T.thermophila* T26S exhibits a marked decrease in binding interactions at protein concentrations greater than 30 μg.

In order to further confirm binding of core proteins to the rRNAs gel shifting experiments were performed [see Materials & Methods]. Naked yeast YTR exists as two conformations a & b in a native RNP gel [fig. 23 lane1], while the same transcript resolved into only one band in a denaturing

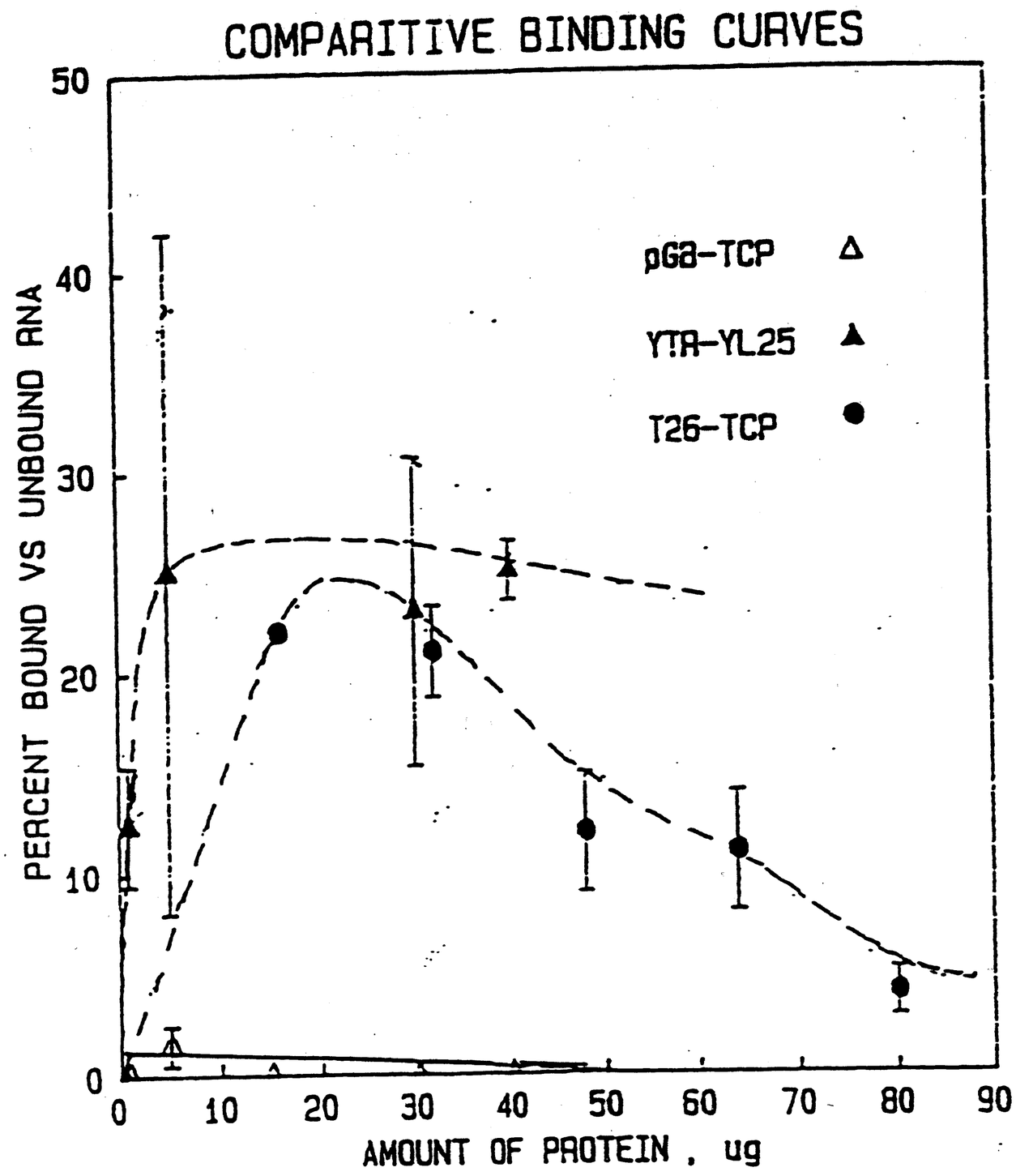

Fig. 20 : BINDING OF *T.thermophila* CORE RIBOSOMAL PROTEIN [TCP] TO HOMOLOGOUS MATURE 26 S rRNA AND PRECURSOR T7 TRANSCRIPT. The percent of bound versus unbound rRNAs were calculated using a nitrocellulose filter binding assay, as described previously. Tetrahymena 26 S rRNA and precursor T7 [pGB] were incubated with its homologous core proteins [TCP]. Yeast SP6 transcript [YTR] with its homologous yeast YL25 protein was used as a positive control for binding. At a confidence level of > 99 %, pGB-TCP was significantly different from YTR-YL25 and T26-TCP while no significant difference was obtained between YTR-YL25 AND T26-TCP.

polyacrylamide gel [fig.24 lane 1]. The *T.thermophila* pGB transcript however, appears as only one conformation in both native RNP and denaturing gels, suggesting that perhaps the pGB transcript forms a less compact structure [figs.23 A]. Limited S1 nuclease mapping of *T.thermophila* pGB transcript has indicated that the precursor pGB transcript is capable of forming some secondary structure. A comparable electrophoretic banding pattern was obtained with limited S1 nuclease for both yeast YTR and *T.thermophila* pGB transcripts [Ware, unpublished results]. How this less compact structure may affect heterologous binding is unknown.

Since the YTR transcript in yeast is known to contain the entire L25 binding site, it was convenient to test the binding interactions of core proteins with YTR in gel shift and filter binding

assays.

The RNP complex of YTR + YL25, at 4 - 8 μg of YL25 , appears to shift electrophoretically, resulting in an equal distribution of RNPs between conformations a & b [see fig.23 lanes 2 - 4]. However at 16 ug of YL25, the RNP appears to shift back to conformation b. When 4 μg of Tetrahymena TCP was used, the RNP shifted to conformation a, although a trailing band at conformation b was still detected. Furthermore, at 8 ug of TCP protein, a significant decrease in conformation a by an increase in conformation b was observed. A subsequent decrease in both conformation a & b was detected at 16 ug of TCP [see

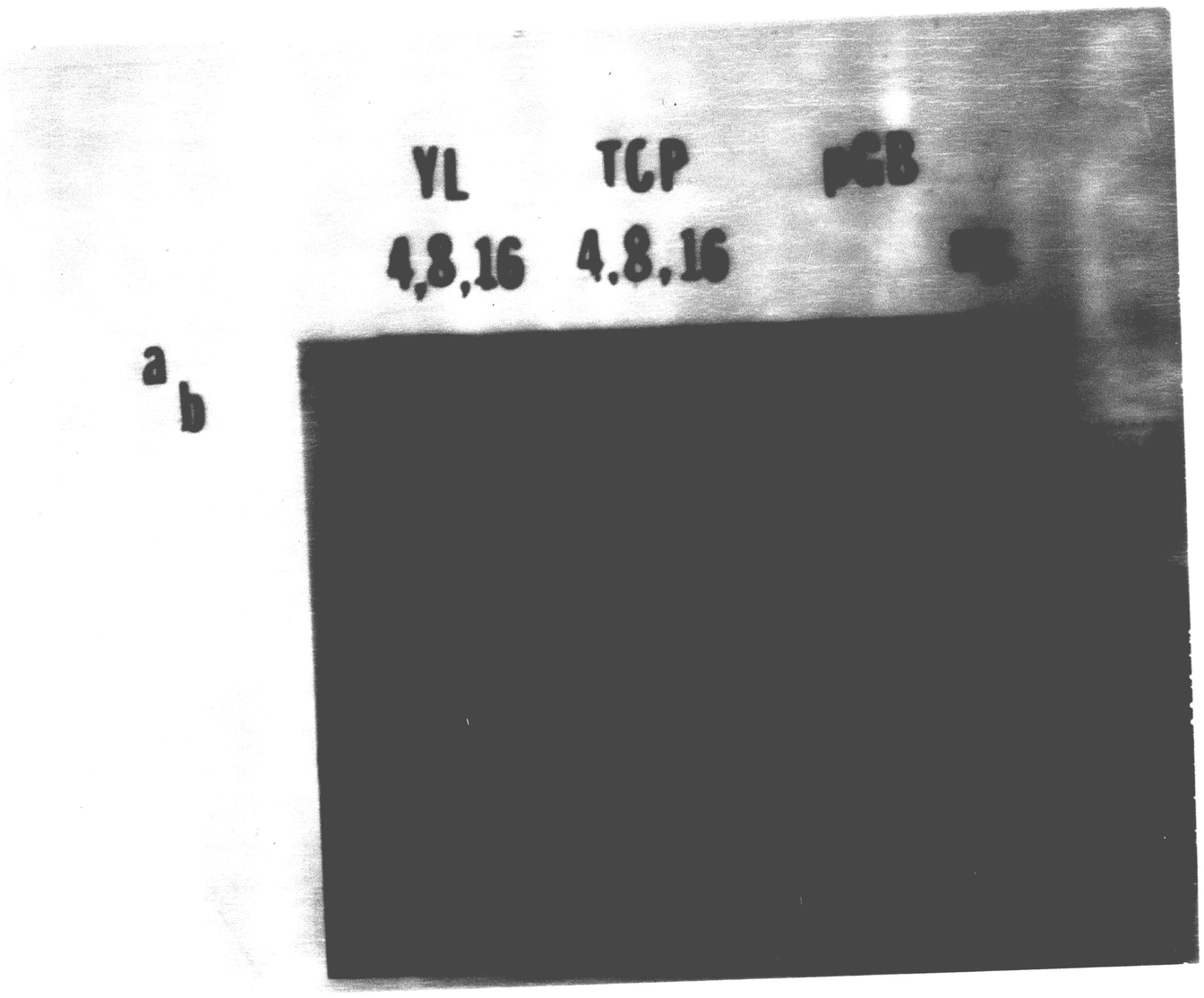

Fig.21 : GEL SHIFT ASSAY OF YEAST SP6 TRANSCRIPT YTR AND *T.thermophila* T7 TRANSCRIPT pGB500 WITH YEAST YL25 AND *T.thermophila* TCP RIBOSOMAL PROTEINS. Equal amounts [50 ng/20cpm/lane] were incubated with varying concentrations of ribosomal proteins YL25 and TCP. Lane 1 : naked YTR rRNA in conformation a and conformation b ; lanes YL : YTR with 4,8 & 16 µg YL25 ; lanes TCP : YTR with 4,8 & 16 µg TCP and lanes pGB : pGB500 with 4,8 & 16 µg TCP.

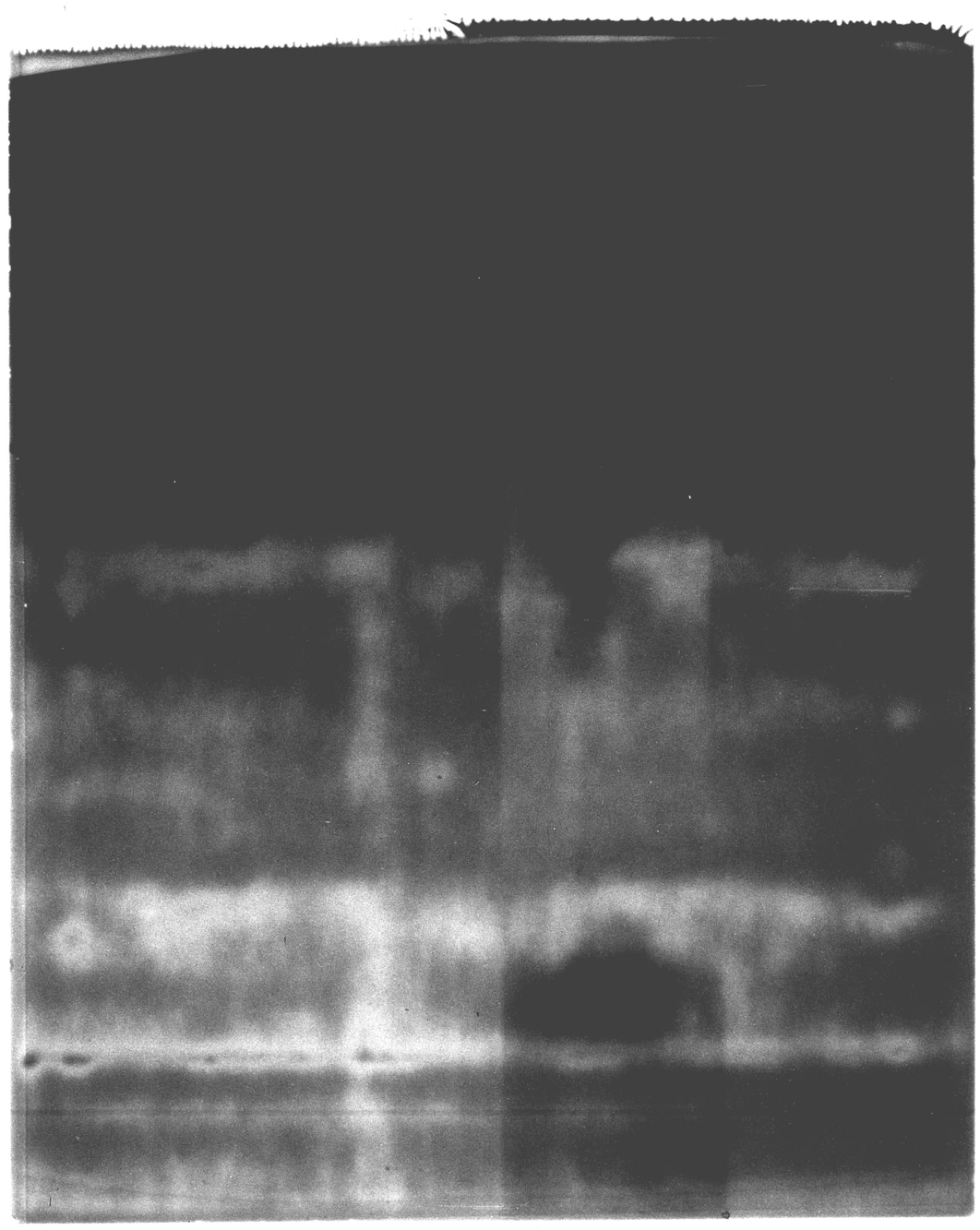

Fig. 22 : DENATURING POLYACRYLAMIDE ELECTROPHORESIS OF YEAST SP6 TRANSCRIPT YTR. Yeast SP6 transcript labelled with ^{32}P was elecctophoresed on a 8 % 7M urea gel and autoradiographed. Arrow denotes a single band representing YTR transcript.

fig.23 lanes 5 - 7]. Some RNA degradation was observed at high protein concentrations. When naked *T.thermophila* precursor T7 transcript [pGB500] was subjected to a gel shift only one conformation was observed. At all concentrations of TCP protein [i.e. 4, 8 & 16 μg], the RNP complex of pGB + TCP did not demonstrate an electrophoretic shift [fig.23, lanes 8 - 11].

This result confirms the nitrocellulose filter binding experiments. The trailing bands at conformation a [Fig.23,lanes 4 & 6] correspond well with the decrease in percent of bound rRNA in the binding curves [Fig.24 B]. These results are more consistent with the hypothesis that something about the structure of the precursor may not foster L25 binding. Whether this is related to the presence of gap bases in the precursor rRNA is unclear at this

time.

CORE PROTEINS PROTECT UNIQUE FRAGMENTS ON 26S rNA

In order to determine whether a candidate for L25 exists among *T.thermophila* core proteins, RNase T1 protection assays were performed as described in Materials & Methods.

Naked *T.thermophila* 26S rRNA demonstrates a definite banding pattern with limited RNase T1. With saturating concentration of core proteins, RNase T1 appears to protect two unique fragments of the 26S rRNA [see fig.25]. These fragments are being extracted out and will be sequenced, in order to determine if an L25 homologue[s] exist among the core proteins.

SUMMARY OF RESULTS

[1] Three 15 Kd core ribosomal proteins were isolate from *T.thermophila*.

[2] Core propteins specifically bind to 26S rRNA and protect two unique fragments of the 26S rRNA.

[3] Core proteins demonstrate preferential binding to mature 26S rRNA while no interaction was observed with the precursor unprocessed pGB transcript.

[4] Core proteins from *T.thermophila* do interact with the yeast YTR transcript in gel shift and filter binding assays, suggesting that a candidate for an L25 homologue does exist among the core proteins.

[5] Core proteims cause an electrophoretic shift with yeast YTR but not with precursor pGB

transcript, implying that differences in binding requirements exist between a gap processing organism [*T.thermophila*] and one that does not [yeast].

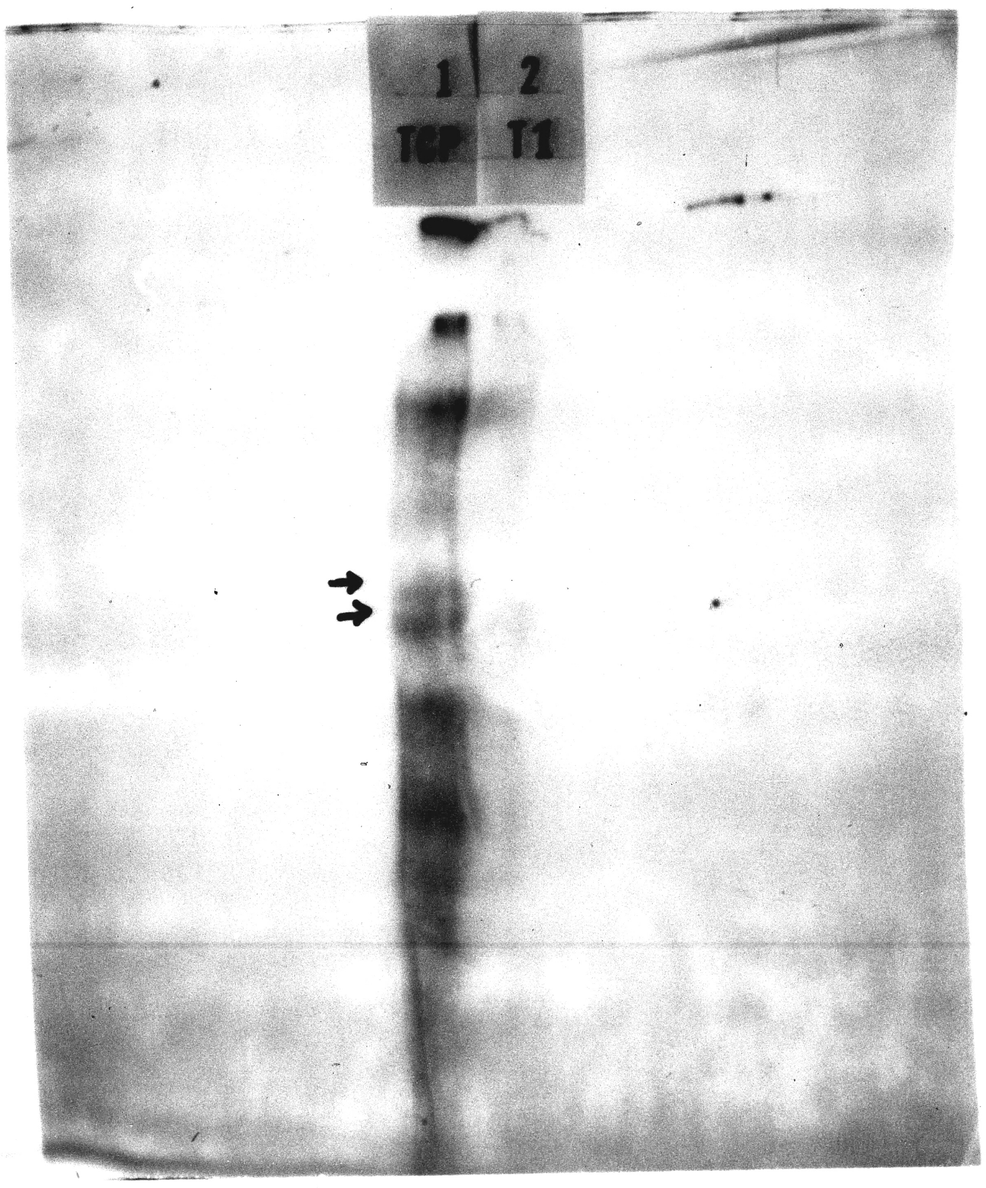

Fig.25 : PROTECTION PATTERN OF *T.thermophila* CORE PROTEINS TCP WITH 26 S rRNA. Pointer denotes the unique fragments protected in the TCP lane.

SECTION II
DISCUSSION

L25 homologues have been isolated from a wide selection of prokaryotic, eubacterial, and archaebacterial organisms , however the only eukaryotic organism from which this ribosomal protein has been isolated has been yeast [El-Baradi et al 1985]. The L25 binding site on the 26S rRNA is interrupted by the presence of the hidden break in insects [*D.melanogaster*] and protozoans [*T.thermophila*]. Previous studies have indicated that yeast YL25 is capable of binding to mature 26S rRNA but unable to bind to the precursor 26S rRNA transcript from *T.thermophila* [Ware, submitted]. This result suggests that perhaps processing in this region of the 26S

rRNA is a prerequisite for L25 binding and subsequent assembly into functional subunits. Based on previous heterologous binding experiments done with E.coli and mouse lr RNAs, it was proposed that the size of the variable V9 region might have a role in L25 binding. However when chimeric lr RNAs, in which the V9 region of yeast was replaced with V9 regions from mouse and Tetrahymena, were used in binding studies, L25 binding was not altered significantly [W. Musters, personal communication]. These results suggest that perhaps there are different binding requirements for L25 in organisms that gap process [*T.thermophila*] and ones that do not [yeast]. In order to investigate these differences, an attempt was made to isolate the L25 homologue from *Tetrahymena thermophila* using extraction methods successful for yeast.

ISOLATION OF CORE PROTEINS

Previous studies have indicated that only one L25 ribosomal protein [20 Kd] remains bound to yeast core particles, after treatment with LiCl [El-Baradi et al 1985]. However using similar extraction methods successful for yeast, *T.thermophila* core particles appear to possess at least three ribosomal proteins, in the 15 Kd range. The core proteins isolated from *T.thermophila* are smaller than their counterparts in yeast. It is quite possible that the L25 homologue[s] in *T.thermophila* may exist in more than one form; that is the function of an L25 homologue[s] in a processing organism may be shared by more than one early binding ribosomal protein. Future work involves further separation of the core proteins by RP-HPLC. Individual proteins will be

tested for their ability to bind to mature and precursor 26S rRNAs.

BINDING INTERACTIONS WITH MATURE AND PRECURSOR rRNAs

The binding inetraction of *T.thermophila* core proteins with 26S rRNA appears to be severely inhibited at excess concentrations of protein [see Fig.22]. This inhibition is not so prominent with the yeast YTR transcript. Inhibition may be due to the presence of more than one early binding ribosomal protein in *T.thermophila* core proteins. It is possible that one ribosomal protein could have a negative effect on the binding of another protein. This possibility will be tested by sequential binding experiments. Indivual core proteins will be

subjected to gel shift assays in order to determine which core protein[s] bring about this inhibitory effect.

Based on sequence conservation at the L25 binding site among prokaryotes and eukaryotes, an *in vitro* precursor *T.thermophila* transcript [pGB], containing the presumptive binding site was prepared. *T.thermophila* core TCP proteins demonstrated a preferential binding to mature processed 26S rRNA, as determined by filter binding assays [see fig.22]. However *T.thermophila* core proteins [TCP] are unable to bind to the precursor [pGB500] transcript. This inability of *T.thermophila* precursor transcript [pGB] to bind to either yeast or *T.thermophila* core ribosomal proteins [fig.22 & 23], suggests that perhaps the pGB transcript may not form the correct secondary structure. Incorrect secondary structure, in gap

processing organisms, could result in inaccesibility of the L25 binding site. Preliminary partial S1 nuclease digestions of *T.thermophila* pGB and yeast YTR transcripts have shown that there is some similarity in secondary structure in both transcripts [Ware, unpublished results]. Therfore, the inability of core proteins to bind to the precursor pGB transcript may not be entirely attributed to secondary structure. However, gel shift assays have shown that *T.thermophila* pGB transcript forms a less compact structure as compared to the yeast YTR transcript. Clearly structure differences exist between yeast and *T.thermophila* transcripts. Whether the presence of unprocessed nucleotides in *T.thermophila* prevent L25 homologue[s] from interacting with pGB transcript, has yet to be determined. However, it is possible that in gap processing organisms like

T.thermophila, sequences outside the central domain III may be required for folding of the precursor rRNA into its correct secondary structure. These results are consistent with the hypothesis that perhaps different binding requirements exist between organisms that gap process [Tetrahymena] and ones that do not [yeast]. This hypothesis will be tested with gel shift assays. The shift of *T.thermophila* core proteins with isolated conformers of the yeast YTR transcript will be tested, in order to determine which conformer is conducive for L25 binding. Alternatively, precursor transcripts containing additional 26S rRNA sequences either upstream or downstream of the gap region in *T.thermophila* will be prepared, in order to ascertain whether binding differences do exist between yeast and Tetrahymena.

T.thermophila core proteins are capable of

interacting with the yeast YTR trancript which contains the entire L25 binding site, as determined by gel shift assays. Preliminary heterologous filter binding experiments have shown that *T.thermophila* core proteins do bind to yeast YTR [78% rRNA was retained on the filter, data not shown]. These two results provide strong evidence that a candidate for an L25 homologue[s] does exist among the core ribosomal proteins. Inability of *T.thermophila* pBG transcript containing the presumptive binding site to interact with either yeast or its homologuos core proteins, may be consistent with the hypothesis that the L25 would bind to the mature rRNA but not to the unprocessed precursor rRNA. These results are also consistent with the hypothesis that differences in binding requirements exist between gap processing organisms [*T.thermophila*] and organisms that do not

[yeast].

The core proteins from *T.thermophila* clearly protect two unique fragments of the 26S rRNA when treated with limited RNase T1. These protected fragments will be extracted and sequenced, in order to determine if an L25 candidate exists among the core proteins.

Once the L25 homologue has been isolated from *T.thermophila*, the protein will be sequenced and characterised by H.Raue [Netherlands]. A *T.thermophila* cDNA library enriched for ribosomal proteins will be screened for the L25 gene , using yeast L25 probes.

It is hoped that the isolation of an L25 homologue from a gap processing organism like *T.thermophila* will aide in studying its involvement in the maturation and assembly of ribosomal subunits.

REFERENCES

Branlant, C.; Krol, A.; Sriwidada, J.; Fellner, P. and Crichton, R. [1973] The identification of the RNA binding site for a 50 S ribosomal protein by a new technique. FEBS Lett. 35:215

Cech , T.R. and Brehm , S.L. [1981] Replication of the extrachromosomal ribosomal RNA genes of *Tetrahymena thermophila* Nucl.Acid. Res. 9 [14] , 3531 - 3543

Cech , T.R. [1986] Ribosomal RNA gene expression in Tetrahymena : Transcription and RNA splicing . The Molecular Biology of Ciliated Protozoans , pp 203 - 225

Cuny , M. ; Milet , M. and Hayes , D. [1982] Proteolytic degradation of ribosomal proteins during isolation of ribosomes and ribosomal subunits from Tetrahymena . Biochimie 64 , 1049 -1058

Darnell , J.E. [1983] Processing of RNA . Scientif. Amer. 90 - 100

Dreisig , H. ; Andreasen , P.H. and Kristiansen , K.

[1984] Regulation of ribosome synthesis in *Tetrahymena pyriformis*

[1] Coordination of synthesis of ribosomal protein and ribosomal RNA during nutritional shift down. FEBS 140 , 469 - 475

[2] Coordinate synthesis of ribosomal protein and ribosomal RNA during nutritional shift up. FEBS 140, 477 - 483

[3] Analysis by translation *in vitro* of RNA isolated during nutritional shift down and nutritional shift up. FEBS 140 , 485 - 492

Edwards , K. and Kossel , H. [1981] The rRNA operon from *Zea mays* chloroplast : Nucleotide sequences of the 23S rRNA and its homology with *E.coli* 23S rDNA . Nucl.Acid. Res. 9 , 2852 - 2869

El-Baradi , T.T.A.L. ; Raue , H.A. ; de Regt , V.C.H.F. and Planta , R.J. [1984] Stepwise dissociation of yeast 60 S ribosomal subunits with LiCl and identification of L25 as a primary 26 S rRNA binding protein . Eur.J. Biochem. 144 , 393 -

El-Baradi , T.T.A.L. ; Raue , H.A. ; de Regt , V.C.H.F. ; Verbee , E.C. and Planta , R.J. [1985] Yeast ribosomal protein L25 binds to an evolutionary conserved site on yeast 26S and *E.coli* 23S rRNA. EMBO J 4 [10] , 2101 -2107

El-Baradi , T.T.A.L. ; van der Sande , C.A.F.M. ; Mager , W.H. ; Raue , H.A. and Planta , R.J. [1986] Cellular level of yeast ribosomal protein L25 is controlled principally by rapid degradation of excess protein . Curr. Genet. 10 , 733 - 739

El-Baradi , T.T.A.L. ; de Regt , V.C.H.F. ; Planta , R.J. ; Nierhaus , K.H. and Raue , H.A. [1987] Interaction of ribosomal proteins L25 from *E.coli* with yeast 26S rRNA and mouse 26S rRNA . Biochimie 69 , 939 - 948

Fujiwara , H. and Ishikawa , H. [1986] Molecular mechanism of introduction of the hidden break into the 28S rRNA of insects : implication based on structural studies . Nucl. Acid. Res. 14 , 6393 - 6401

Garrido , S. ; Gonzalez , P.J. ; Palacian , E. and Hernandez , F. [1990] Reconstitution of rat liver 60

S ribosomal subunits following disassembly by dimethylmaleic anhydride.Molec. Cell. Biochem.92,159- 167

Garrett , R.A. ; Rak , K.H. ; Daya , L . and Stouffler , G. [1971] Ribosomal proteins : Specific protein binding sites on 16 S rRNA of *E.coli* . Molec. Gen. Genet. 134 , 112- 124

Ishikawa , H. [1977] Evolution of ribosomal RNA . Comp. Biochem. Physiol. 58 B , 1 - 7

Kruiswijk , T. ; Planta , R.J. and Krop , J.M. [1978] The course of the assembly of ribosomal subunits in yeast . Biochim. Biophys. Acta. 517 , 378 - 389

Lansverin , G.de. and Jacq , B. [1989] Sequence and secondary structure of the Central Domain of Drosophila 26 S rRNA : Universal model for the Central Domain of the large rRNA containing the region in which the central break may happen . J. Mol. Evol. 28 , 403 - 417

Leer , R.J. ; van Raamsdonk-Duin , M.M.C. ; Hagendorn , M.J.M. ; Mager , W.H. and Planta , R.J. [1984] Structural comparison of yeast ribosomal protein genes . Nucl. Acid. Res. 12 [17] , 6685 -

Mager , W.H. [1988] Control of ribosomal protein gene expression . Biochim. Biophys. Acta. 949 , 1 - 18

Mankin , A.S. and Kagramanova , V.K. [1986] Complete nucleotide sequence of the single ribosomal RNA operon of *Halobacterium holobium* : secondary structure of archaebacterial 23 S rRNA . Mol. Gen. Genet. 202 , 152 - 161

Michot , B. and Bachellerie , J - P. [1987] Comparison of large subunit rRNAs reveal some eukaryotic specific elements of secondary structure. Biochimie 69 , 11 - 23

Nieto , M.A. ; Hernandez , F. and Palacian , E. [1989] Disassembly and reconstitution of yeast 60S ribosomal subunits . Molec. Cell. Biochem. 86 , 55 - 63

Petridou , B. ; Cuny , M. ; Guerin , M - F and Hayes, F. [1983] Ribosomal subunits and ribosomal proteins of *Tetrahymena thermophila* : Effect of the presence of iodoacetamide during ribosome extraction on the properties of the subunits . Eur. J. Biochem. 135 , 425 - 434

Petridou , B. ; Guerin , M - F and Hayes , F. [1989] Protein - RNA cross-linking in the subunits of cytoplasmic ribosomes of *Tetrahymena thermophila* . Biochimie 71 , 667 - 679

Petridou , B. ; Guerin , M - F. and Hayes , F. [1989] Ribosomal proteins of *Tetrahymena thermophila* : Correlation of one - and two - dimensional electrophoretic migration patterns and characterization of additional small and large subunit proteins. Biochimie 71 , 655 - 663

Raue , H.A. ; El-Baradi , T.T.A.L. and Planta , R.J. [1985] Evolutionary conservation of protein binding sites on high-molecular-mass ribosomal RNA . Biochem. Biophys. Acta. 826 , 1 - 12

Rodrigues - Pousada , C. and Hayes , D.H. [1978] Ribosomal subunits from *Tetrahymena pyriformis* : Isolation and properties of active 40 S and 60 S subunits . Eur. J. Biochem. 89 , 409 - 415

Rubin , G.M. [1975] Preparation of RNA and ribosomes from yeast . Methods in Cell Biology . v.xi , 45 - 64

Seilhammer , J.J. ; Gutell , R.R. and Cummings ,

D.J. [1984] Paramoecium mitochondrial genes II : large subunit rRNA gene sequence and microevolution. J. Biol. Chem. 259 , 5173 - 5181

Spangler , E.A. and Blackburn , E.H. [1986] The nucleotide sequence of the 17 S rRNA gene of *Tetrahymena thermophila* and the identification of point mutations resulting in resistence to the antibiotic puromycin and hygromycin . J. Biol. Chem. 260 [10] , 6334 - 6340

Stern , S. ; Powers , T. ; Changchien , L - M . and Noller , H.F. [1989] RNA-protein interactions in 30S ribosomal subunits : Folding and function of 16 S rRNA . Science 244 , 783 - 790

Sutton , C.A. and Hallberg , R.L. [1979] Ribosome biosynthesis in *Tetrahymena thermophila* : Regulation of ribosomal RNA degradation in growing and growth arrested cells . J. Cell. Physiol. 101 , 349 - 358

Tollervey , D. [1987] A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. EMBO J 6 [13] , 4169 - 4175

Tollervey , D. ; Fournier , M.J. and Zagorski , J. [1988] Characterisation of an SNR gene locus in *Saccharomyces cerevisiae* that specifies both dispensable

and essential small nuclear RNAs . Molec. Cell. Biol. 8 [8] , 3282 - 3290

Trout , R.R. ; Hermack , R.L. ; Sun , T - T. ; Hershey , J.W.B. and Bollen , A. [1974] Protein topography of ribosomal subunit from *E.coli* ribosomes. Ribosomes [Ed. Nomura et al] , 271 -308

Vester, B. and Garrett, R.A. [1984] Structure of a protein L23-RNA complex located at the A site Domian of the ribosomal peptidyl transferase centre. J.Mol.Biol. 179: 431 - 452

Ware , V.C. ; Renkawitz , R. and Gerbi , S.A. [1985] rRNA processing : removal of only nineteen bases at the gap between 28 S α and 28 S β rRNAs in *Sciara coprophila* . Nucl. Acid. Res. 13 [10] , 3581 - 3597

Warner, J.R.; Kumar, A.; Udem, S. and Wu, R.S. [1973] Ribosomal proteins and the assembly of ribosomes in eukaryotes. Biochem. Soc. Symp. 37 : 3

Warner , J.R. [1975] Assembly of ribosomes in eukaryotes. Ribosomes [Ed. Nomura et al] , 461 - 488

Warner , J.R. [1989] Synthesis of ribosomes in *Saccharomyces cerevisiae*. Microbiol. Rev. 53 [2] , 256 -

Wool , I.G. and Stoffler , G. [1975] Structure and function of eukaryotic ribosomes . Ribosomes [Ed. Nomura et al] , 417 - 460

Woudt , L.P. ; Mager , W.H. ; Beek , J.G. ; Wassenaar , G.H. and Planta , R.J. [1987] Structural and putative regulatory sequences of the gene encoding ribosomal protein L25 in *Candida utilis* . Curr. Genet. 12 , 193 - 198

Woudt , L.P. ; Mager , W.H. ; Smit , A.B. and Planta, R.J. [1987] Conserved sequence elements upstream of the gene encoding yeast ribosomal protein L25 are involved in transcriptional activation . EMBO J 5 [5] , 1037 - 1040

Xiang , R.H. and Lee , J.C. [1989] Identification of proteins cross-linked to RNA in 40 S ribosomal subunits of *Saccharomyces cerevisiae* . Biochimie 71 : 1201 - 1204

Xiang , R.H. and Lee , J.C. [1989] Identification of neighbouring protein pairs in the 60 S ribosomal subunits from *Saccharomyces cerevisiae* by chemical cross-linking . J. Biol. Chem. 264 [19] , 10542 - 10546

Yao , H - C . [1986] Amplification of ribosomal RNA genes. The Molecular Biology of Ciliated Protozoans, 203 - 225

Zimmermann , R.A. [1974] RNA -protein interactions in the ribosome . Ribosomes [Ed. Nomura et al] , 225 - 269

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